# MOLECULAR ANALYSIS OF QUINOLINE METABOLISM IN *PSEUDOMONAS AERUGINOSA* QP

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Cellular and Molecular Biology in the University of Canterbury.

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To my father, Tony (1938-96), who first placed a spanner in my hand and encouraged me to wonder: *how*?

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# ABSTRACT

Quinoline is a toxic nitrogenous heterocyclic compound that accumulates in the environment as a result of incomplete combustion of fossil fuels. Microbiological degradation of quinoline is an important mechanism in its removal. A *Pseudomonas aeruginosa* strain with this ability was isolated previously. This strain (QP) was transposon mutagenised to isolate quinoline-degradation deficient mutants ($Qln^-$). Putative mutants were isolated using a two-step screening process. Mutants failing to grow on minimal salts agar plates supplemented with quinoline as the sole source of carbon, nitrogen and energy were subsequently incubated in an analogous liquid medium. UV spectrophotometry of the cell free media was used to confirm their stability, as quinoline has a characteristic UV absorption spectrum in aqueous solution, any change in this spectrum revealed a modification to the molecule. Two of the five isolates judged $Qln^-$ following the plate assay, grew in the quinoline/minimal salts liquid medium with concomitant loss of quinoline, albeit slower than the wild type parent and were not studied further. The three remaining mutants could not grow in quinoline media without supplementary glucose. The UV spectra of cell-free media (containing both glucose and quinoline) taken from cultures of the remaining 3 $Qln^-$ mutants were identical. Spectral data suggested that the 3 mutants accumulated a single compound, this was identified by NMR analysis as 8-hydroxycoumarin. In all three $Qln^-$ mutants, the transposon had inserted into the same 8.6 kb *Bgl*II fragment of the genome. This *Bgl*II fragment containing the transposon insertion was cloned (pNHQ8) and a restriction map generated. Using subclones generated from pNHQ8 it was possible to screen a library of restriction fragments from the wild-type strain and clone the wild-type fragment. This fragment was extended using uninterrupted sequences from the mutant strain. The full size fragment could

complement the Qln⁻ mutants back to the quinoline degrading phenotype. The complementing plasmid was used to transform a non-quinoline degrading *P. aeruginosa*. This transformant could modify 8-hydroxycoumarin to a compound tentatively identified as 2,3-dihydroxyphenylpropionic acid, indicating that this plasmid encoded an enzyme. From these clones, approximately 3 kb of DNA sequence data was generated.

A previous study of *P. aeruginosa* QP indicated that quinoline degradation was associated with four large plasmids. However, this study failed to isolate any plasmid DNA from this organism or connect the quinoline degrading phenotype with plasmids through a variety of techniques.

During the course of the study it was observed that *P. aeruginosa* QP strains exhibited a high degree of DNA restriction, which could be partially overcome by growing the organism at 43 °C. It was also observed that the organism rapidly degraded chromosomal DNA upon cell lysis. It was hypothesised that QP strains produced exonucleases. Both these characteristics provided challenges to the genetic manipulation of this organism.

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| | |
|---|---|
| $A_{600}$ | absorbance at a given wavelength (eg 600 nm) |
| aa | amino acid |
| AGE | agarose gel electrophoresis |
| BLAST | basic local alignment search tool |
| BHN | brain heart nitrate broth |
| BHS | Bushnell-Haas minimal media (no N or C source) & trace elements |
| bp | base pair |
| BSA | bovine serum albumin |
| cfu | colony forming units |
| CHEF | contour-clamped homogeneous electric field (gel electrophoresis) |
| cpm | counts per minute |
| $dH_2O$ | sterile distilled water |
| ds | double stranded |
| EDTA | ethylenediaminetetra-acetic acid |
| hr | hour(s) |
| IPTG | isopropyl-β-D-thio-galactopyranoside |
| kb | kilobase pairs |
| Mb | mega base pairs |
| min | minute(s) |
| N | any nucleotide (G, A, T or C) |
| NCBI | National centre for biotechnology information |
| o/n | overnight (typically 14-18 hours) |
| ORF | open reading frame |
| PAH | polycyclic aromatic hydrocarbon |
| PMSF | phenylmethylsulfonylfluoride |
| QBHS | BHS amended with 0.03% v/v quinoline |
| $Qln^-$ | bacteria unable to grow on quinoline |
| $Qln^+$ | bacteria able to grow on quinoline |

| | |
|---|---|
| rpm | revolutions per minute |
| SDS | sodium dodecyl sulphate |
| sec | second(s) |
| ss | single stranded |
| TEMED | N,N,N',N',-tetra-methylethylenediamine |
| X-Gal | 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside |

# 1. INTRODUCTION

Heterocyclic structures form a part of up to two-thirds of the estimated 4 million known organic structures (Kuhn and Suflita 1989). In biological systems these heterocyclic aromatics exist as electron carriers, nucleotides, energy storage molecules, mycotoxins and alkaloids and perform many other functions. Humanity, in industry, uses heterocyclic structures for solvents, dyes, explosives, pharmaceuticals and pesticides. By-products from the mining industry, coal-tar and oil shale processing, wood-preserving facilities and chemical manufacturing plants also contain large quantities of heterocyclic aromatic compounds (Kaiser et al. 1996). Anthropogenic use and processing of these compounds has led to their mobilisation into the environment.

Heterocyclic compounds have been shown in numerous studies to be significantly mutagenic in the Ames histidine reversion assay and to be carcinogenic and tumorgenic to mice, hamsters and rats (Santodonato and Howard 1981). The aqueous solubility of heterocyclic compounds is enhanced by their structure when compared to their homocyclic analogues and this coupled with their weaker sorption to soil and aquifer organic material, makes their transport through groundwater systems of greater concern (Kuhn and Suflita 1989). These facts have raised the concern for the presence of these compounds in the environment and the associated health hazard for humans and wildlife.

Losses of these compounds in the environment can be broadly classified into two categories: biotic; and abiotic processes. Biotic processes (biodegradation) have the potential to remove heterocyclic compounds from the environment but many criteria must be met. An appropriate microbial community possessing the appropriate catabolic potential must be present. Environmental parameters must be conducive to the appropriate organism(s). Bioavailability of substrate for appropriate microbial interaction must also be considered (Mueller et al. 1989).

It is therefore necessary to gain a clearer understanding of the processes by which these compounds are degraded and the types of microorganisms involved (Kimura and Omori 1995; Kaiser et al. 1996).

Quinoline is a nitrogen heterocycle (azaarene) consisting of fused pyridine and benzene aromatic ring structures. Quinoline has been shown to be mutagenic on *Salmonella typhimurium* tester strains (Nagao et al. 1977), carcinogenic in rats when administered orally (Hirao et al. 1976) and to covalently bind to nucleic acids (Tada et al. 1980). It is believed to be the most abundant environmental azaarene carcinogen (Boyd et al. 1987).

Quinoline has been found to be degraded in situ by various bacteria in several studies (Kimura and Omori 1995). In vitro studies have revealed 2 separate, incomplete pathways of aerobic quinoline degradation by bacteria (Shukla 1989; Schwarz et al. 1989). In this study molecular techniques were used to investigate quinoline metabolism in *Pseudomonas aeruginosa* QP.

## 1.1 AZAARENES, QUINOLINE AND THE ENVIRONMENT

### 1.1.1 Structure and physical properties of quinoline and related compounds

Quinoline (benzo[b]pyridine) belongs to a family of chemicals known as polycyclic azaheterocyclic compounds (azaarenes). Azaarene compounds are N-heterocycles, they are composed of a 5 or 6-membered ring structure containing a nitrogen atom, this ring may be fused to one or more aromatic ring structures. As such, these compounds can be considered nitrogen containing analogues of the polyaromatic hydrocarbons (Santodonato and Howard 1981). The structure of all the azaarenes may be described as derivatives of the following six ring systems: pyridine, quinoline, isoquinoline, acridine, carbazole and phenanthridine (Figure 1-1).

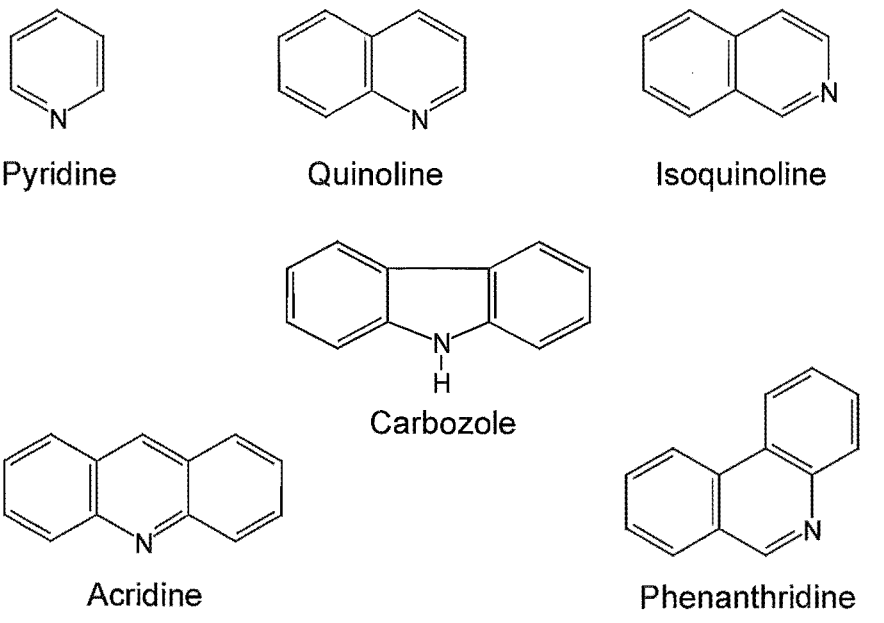


**Figure 1-1. Structures of the parent azaarenes.**

Heteroaromatic structures are more polar than their homocyclic homologues, the higher water solubility of these compounds is due to the formation of a hydrogen bond with the lone pair of electrons on the nitrogen atom (Pereira et al. 1983). Heteroaromatics possess lower octanol/water partition coefficients ($K_{ow}$), this low $K_{ow}$ is associated with the higher water solubilities of these compounds and their weaker sorption to soil and aquifer organic material (Kuhn and Suflita 1989).

Quinoline is a liquid at room temperature, colourless (but turns brown on exposure to light) with a characteristic sweet odour (Elderfield 1952). The boiling point at 760 mm of mercury is 237.7 °C and the freezing point is -15.6 °C. Quinoline has a density of 1.094 at 20 °C. Quinoline is sparingly soluble in water, its solubility has been found to be 6.11 g/l (at 20 °C) by infrared measurements and 9.1 g/l (at 19.1 °C) (Jones 1977). Quinoline has a characteristic UV spectrum in water (Figure 1-2). Recorded peaks in absorbance occur at 232, 275, and 315 nm (Jones 1977).

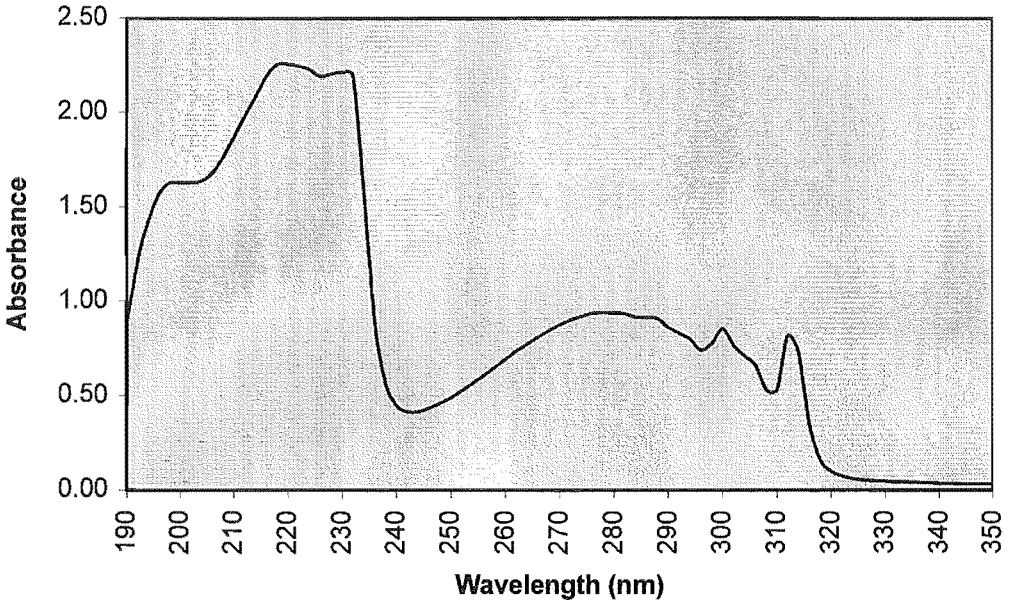


**Figure 1-2. UV spectrum of quinoline in water at neutral pH.**

### 1.1.2 Environmental sources and distribution of azaarenes

Natural sources of azaarenes in the environment include the fossil fuels: coal and coal tar, oil shale and crude oil (Santodonato and Howard 1981), (Schmitter and Arpino 1985) and (Kimura and Omori 1995). Quinoline skeletons are also found in many alkaloids isolated from plants (cigarette and marijuana smoke contain azaarenes (Schmitter and Arpino 1985)), animals, fungi and bacteria (Tibbles et al. 1989). A large percentage of *N*-heterocyclic compounds occurring in the environment are of anthropogenic origin (Schwarz and Lingens 1994).

Oil shale retorting and coal liquefaction industries mobilise these nitrogen heterocycles into the environment, particularly groundwater. Azaarenes are formed during the combustion or pyrolysis processes involving fossil fuels, in a process presumed to be similar for that proposed for PAH compounds where stepwise free radical reactions occur. The source of the nitrogen is not exactly known but is believed to be present in the fuel (Santodonato and Howard

1981). Pereira et al. (1983) suggested that these compounds are pyrosynthesised during the destructive distillation of coal from nitrogen containing compounds such as plant alkaloids or plant pigments that contain the porphyrin skeleton. It is believed that azaarene pollution will become more important as countries such as the United States begin to rely on coal combustion and synfuel derived from fossil fuels (Santodonato and Howard 1981; Pereira et al. 1983).

The increasing cost and scarcity of light petroleum has led to the use of technologies such as solvent refined coal or the conversion of distillation residues. These fuels are high in *N*-heteroaromatic compounds (Schmitter and Arpino 1985). Coal and shale (1.4 % azaarene content) have higher nitrogen contents than standard crude oil, coal derived oil has an azaarene content as high as 9.5 % (Santodonato and Howard 1981). Water effluents from coal coking and other coal processing facilities also contain quinolines at 1-100mg/L as well as higher molecular weight azaarenes (Santodonato and Howard 1981).

Coal carbonisation involves the heating of coal in the absence of oxygen to produce coke, tar and tar-based chemicals and gaseous fuel. A by-product of the coal carbonisation is an ammoniacal liquid which contains these organic bases amongst many other pollutants (Pandey and Handa 1997). The Waikato coal carbonisation plant at Rotowaro had operated for 80 years and for most of that time had discharged its liquid wastes into a nearby river and lake. In the last 10 years of its operation the company was stopped from this dumping practice and stored its liquid wastes on site. When the company went bankrupt, 6.5 million litres of toxic phenolic liquid compounds remained in leaking holding ponds polluting nearby streams and lakes (Stevenson 1991).

Human use of azaarenes includes pesticides, antioxidants, sweetening agents, explosives, dyes and pharmaceuticals. Entry of azaarenes into the soil, atmosphere, groundwater, sediments and aquatic environments has occurred

as a result of these industries (Kimura and Omori 1995). Quinoline has been applied directly into the environment as an insecticide (Schwarz et al. 1988).

Toxic wastes such as coal tar residue also have been buried as a means of disposal, the result has been mobilisation of the components into surrounding groundwater. For example, a single truckload of this waste was dumped in a forest in the northwest of the United States, 30 years later a plume of contamination in the groundwater has spread downstream several hundred metres (Madsen et al. 1991).

A major source of quinoline pollution has entered the environment from the wood preservation industry, approximately $4.5\times10^7$ kg of creosote is consumed annually by creosoting operations in the United States. Creosote is a complex mixture of 85% polyaromatic hydrocarbons, 10% phenolics and 5 % O-, N-, S-containing heterocycles. In the United States it has been estimated that there are 700 sites where creosote wood treatment plants are in, or have been in operation. It has been assumed that the number of creosote contaminated sites approaches the total number of treatment sites due to leaking tanks, drippings from treated timber, spills and leachate from holding ponds being commonplace in this industry (Mueller et al. 1989).

Evidence of azaarenes reaching the environment from anthropogenic origin has been the discovery of these compounds in recent sediments, with a positive correlation between azaarene concentration and proximity to urban areas (Schmitter and Arpino 1985). Azaarenes were detected in the recent sediments of Lakes Zurich and Lucerne, but were undetectable in the deeper sediments which represented the time before the industrial revolution. Lake Zurich had the greatest levels of azaarenes in its recent sediments which was correlated to its more heavily populated and industrialised drainage basin. Natural fires did not seem to contribute toward the accumulation of azaarene compounds in these environments as they were not detected in the older sediments.

The azaarene composition of street dust and European atmospheric particles is similar to that of the polluted sediments which points to these being a significant source. These observations support the assertion of the anthropogenic origin of these azaarenes in the sediments (Wakeham 1979). Sediments at two different locations in Massachusetts were analysed for the presence of azaarenes: a site at Buzzard Bay contained 0.14 mg/kg of azaarene compounds; and a marsh site at Wild Harbour River contained 0.22 mg/kg (Santodonato and Howard 1981).

Azaarenes can also be found in urban suspended particulate matter, car exhausts, engine oil and water from industrialised areas, as they are often formed as a result of incomplete combustion (Schmitter and Arpino 1985). Urban air particles have been tested and found to contain 0.01 to 0.5 ng/m$^3$ of individual azaarene compounds, quinoline has been found at a concentration of 0.6 ng/m$^3$ in Rome (Santodonato and Howard 1981).

The pollution of groundwater is a concern due to its importance as a drinking water resource as, for example, approximately 50% of the US drinking water is supplied from groundwater (Pereira et al. 1983). Industrial waste waters are generally dispensed on land surface, stored on surface impoundments or injected into underground wells (Pereira et al. 1987), liquids like coal tar and creosote are denser than water and percolate down with flow of groundwater (Pereira et al. 1983). Once released, these heterocyclic pollutants are rapidly transported to terrestrial subsurface and can contaminate vast groundwater reserves (Kuhn and Suflita 1989), this process is enhanced, when compared to that of the PAH compounds, by the greater solubility of these heterocyclic compounds (Pereira et al. 1983).

Two examples of sites where industry has caused pollution of their local groundwaters with azaarene contamination have been examined (Pereira et al. 1983; Pereira et al. 1987). Coal tar distillation and wood preservation were activities performed at a site in St Louis Park Minnesota (United States) from 1918-72. When creosote is mixed with water two phases result: a hydrocarbon phase and an aqueous phase which is enriched with phenolic compounds (45%) and heteroaromatics (38%) (Arvin et al. 1989). Percolation of wastewater into groundwater occurred through a thin unsaturated zone, from ponds used to store waste water and a well drilled to a depth of 277m. In 1932 a well for drinking water was drilled at a distance of 1000 m from the plant site, but was closed due to the water having a coaltar taste. Seven other municipal wells have been closed in the region due to contamination. Water extracted from a well 200 m from the site yields a liquid which separates into an aqueous phase and an oily tar phase. The aqueous phase contained many different nitrogen heterocycles of up to 4 rings, the oily phase contained azaarenes with 5 to 12 rings (Pereira et al. 1983). A wood treatment plant near Pensacola, Florida, which operated from 1902-81, discharged wastes into unlined surface impoundments. Two and three ring azaarenes (with $k_{ow}$<3.5) were found in groundwaters (again compounds with greater than 3 rings could not be found in the aqueous phase), total nitrogen heterocycles were as high as 27 mg/L from one sample borehole at a depth of 30 m (Pereira et al. 1987).

Compounds of anthropogenic origin in deep groundwater maybe more refractory than on the surface, as processes such as photodegradation, volatilisation, chemical oxidation or aerobic biodegradation (when oxygen is limiting) do not take place (Pereira et al. 1983). Indigenous organisms in underground aquifers may serve to exacerbate the pollution problems of this resource. Bioconversion of quinoline has been shown to occur under anaerobic conditions in underground aquifers (Pereira et al. 1987) and (Brockman et al. 1989) but at a slower rate than under aerobic conditions. Oxygenated methyl derivatives of quinoline can be produced under these conditions (Figure 1-10), which appear to be biorefractory but have increased

mobility in the aqueous phase due to their greater solubility (Pereira et al. 1987).

### 1.1.3 Biological activity of azaarenes

Numerous azaarenes have been shown to display mutagenic effects in the Ames *Salmonella* histidine reversion system. Samples from cigarette smoke condensate, coal liquefaction products, shale oil and airborne particles have shown to be mutagenic in this test, much of the mutagenicity in each case can be attributed to the azaarene component of these toxic mixtures (Santodonato and Howard 1981). Mutagenesis is taken as strong presumptive evidence for carcinogenesis because of the strong relationship between the molecular events for both these processes. Carcinogencity has been shown for the basic fraction extracted from urban air particles in New York city when injected into newborn mice. Benzacridine derivatives (particularly benz[c]acridine), benzocarbazole derivatives, quinoline and the quinoline derivatives 4-nitroquinoline *N*-oxide and 4-hydroxyaminoquinoline 1-oxide have all been shown to be carcinogenic (Santodonato and Howard 1981).

Quinoline (and many quinoline derivatives including 4-, 6-, 7- and 8-methyl quinoline, 8-hydroxyquinoline and 2,4-dihydroquinoline) were shown to be mutagenic in the Ames mutation assay. All of the quinolines (except 6-nitroquinoline) required activation by the S-9 mix (rat liver preparation) before causing histidine reversion in the *Salmonella typhimurium* tester strains TA100 and TA98 (Nagao et al. 1977). In general, metabolic activation of azaarenes seems to be required for expression of mutagenic activity (Santodonato and Howard 1981).

#### *1.1.3.1 Toxicity of quinoline in mammals*

The effect of various concentrations of orally administered quinoline in rats was monitored (Hirao et al. 1976). The rats were fed a diet supplemented with quinoline at the following levels 0.05%, 0.1% and 0.25% for a period of up to 40

weeks. Most rats treated with the highest concentration of quinoline died within the 40 weeks due to the toxic effects of the chemical or rupture of vascular tumours of the liver. Two types of malignant tumour and nodular hyperplasia developed in the rats liver. These observations were quinoline concentration dependant, nodular hyperplasia and heptocellular carcinoma were seen at the lower quinoline concentrations while hemangioendothelioma was more prevalent at the higher quinoline concentrations. It was presumed that a potent quinoline metabolite produced by the liver was responsible for oncogenic changes in the sinusoidal lining cells or small capillary endothelial cells of the liver.

It was shown for quinoline to be mutagenic, bioactivation by liver enzymes must first occur (Hollstein et al. 1978), formation of quinoline 2,3-epoxide was suggested to be likely. Tada et al. (1980) proposed that the cytochrome P-450 and/or P-448 monooxygenase system was involved with the covalent binding of quinoline to nucleic acids. The proposed means of the metabolic activation of the pyridine ring of quinoline can be seen in Figure 1-3.

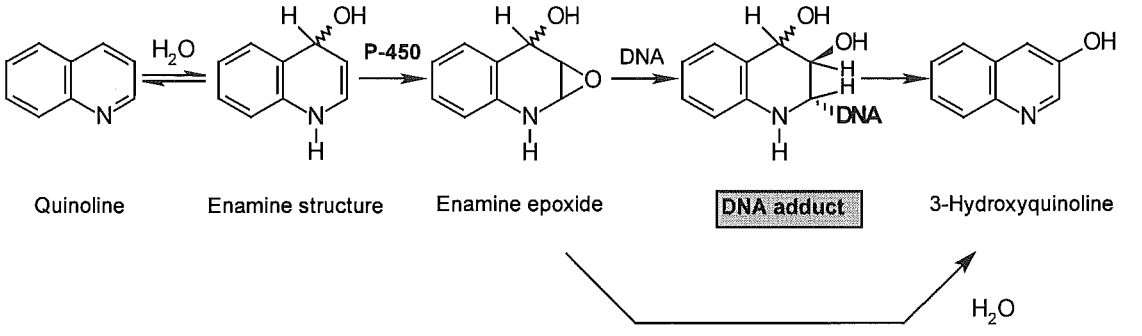


**Figure 1-3. Proposed metabolic activation pathways of quinoline (enamine epoxide theory).** From Saeki et al. (1997).

This theoretical basis for this method of activation was strengthened when the carcinogenicity of 3- and 5-fluoroquinoline were compared (Saeki et al. 1997). Substitution of fluorine at position 3 on the pyridine ring deprived quinoline of its carcinogenicity, while substitution at position 5 resulted in a potent carcinogen. It was suggested that a fluorine at position 5 would stabilise the enamine

structure through hydrogen bonding with the OH at position 4, resulting in a more ready formation of the toxic 2,3-epoxide. Conversely an F-atom at position 3, because of its electron withdrawing effect, might inhibit epoxidation of the 1,4-hydrated enamine structure by preventing the one-electron-step oxidation of the 2,3-double bond.

### 1.1.4 Mechanisms of removal of quinoline from the environment

Figure 1-4 is a diagrammatic summary of natural abiotic and biotic processes of quinoline transport and loss from the environment.

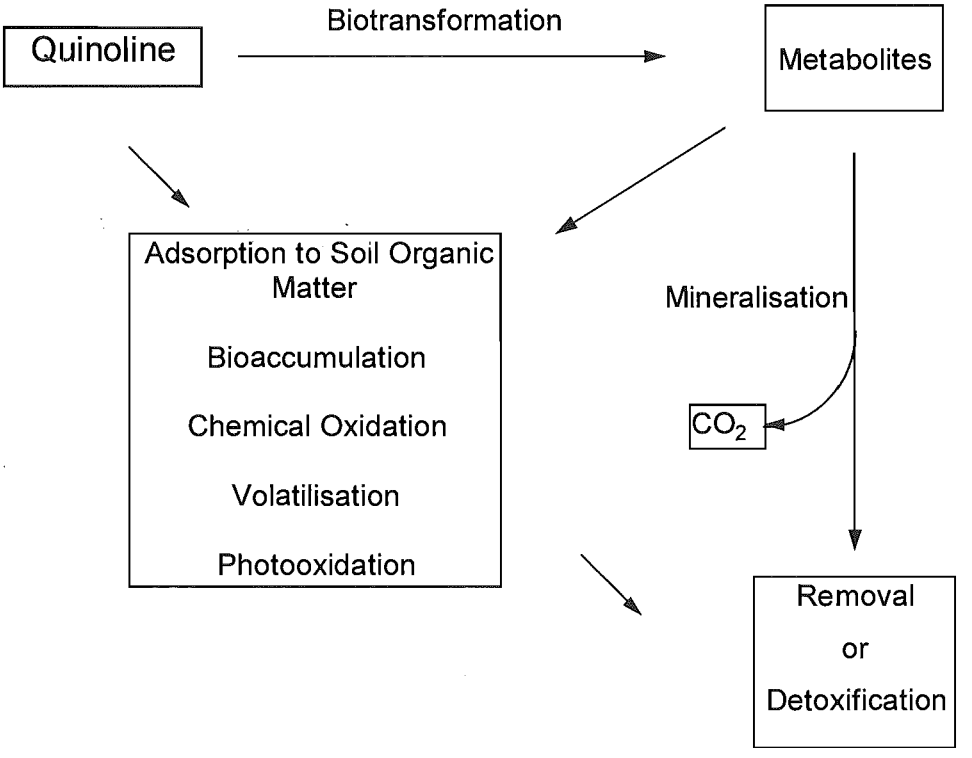


**Figure 1-4. Fate of quinoline in the environment.** Adapted from Cerniglia (1993).

#### *1.1.4.1 Abiotic processes of quinoline loss*

Toxic pollutants may be lost from a polluted site by a number of non-biotic means, such as evaporation, leaching or adsorption onto organic material (Mueller et al. 1989). These processes should only be considered as

displacement mechanisms as the pollutants remain unchanged and are simply transferred to another location in an environment.

Photodegradation of quinoline in water has been studied (Kochany and Maguire 1994; Pichat 1997). Kochany and Maguire (1994) studied the degradation of quinoline in several water samples under both artificial light (313 nm) and sunlight (summer and winter at 40°N latitude). The half life of quinoline in lake water under artificial light was 23 hours, this was reduced to 15 hours at pH 4.5, addition of $NO_3^-$ (0.5mM) or dissolved organic matter at 10 mg/l (DOM) reduced the half life to 10.3 and 9.4 hours respectively. In sunlight, the half life of quinoline was 7.1 days (14 calender days) in summer and 46 days (123 calender days) in winter. Addition of either $NO_3^-$ (0.5mM) or DOM again had a marked effect, reducing both the summer and winter half lives by more than 50%. It was thought that the addition of $NO_3^-$ or DOM may have produced hydroxyl radicals which speeded up the rate of quinoline degradation. 2- and 8-hydroxyquinoline were found to be transient photoproducts of these reactions, prolonged light exposure resulted in destruction of the aromatic nucleus.

Pichat (1997) investigated the effect of UV in the presence of a $TiO_2$ catalyst on several aromatic and alicyclic pollutants in water to aid the understanding of such processes for the treatment of polluted water. Quinoline was studied as a model compound and was found to be degraded by this process. They concluded that the benzene ring could be predominantly attacked by a hydroxy radical, but electrons donated from quinoline to $TiO_2$ could form cation radicals, these would react with superoxide radicals (produced by photocatalysis) forming an oxirane on the pyridine ring which ultimately further degrades.

### *1.1.4.2 Biotic processes of quinoline loss*

Microbial degradation of quinoline and related azaarenes is one of the most important factors in reducing their concentration in the environment (Kimura and Omori 1995). Though many bacteria have been isolated from polluted sites capable of degrading quinoline (Kaiser et al. 1996) (Table 1-1, illustrates a

selection), only a handful of studies report evidence of in situ biodegradation of quinoline.

During a study of groundwater in an aquifer contaminated from a nearby wood treatment facility it was found that both 2(1H)quinolinone and 1(2H)isoquinolinone were in the anaerobic zones of the aquifer (Pereira et al. 1987). These compounds are not normally found in wood-preservative waste water and were assumed to be a result of microbial action. In addition to these hydroxylated compounds, several isomers of their methylated and methoxy derivatives were identified suggesting further microbial transformation. The results of in vitro experiments with a microbial consortia isolated from the aquifer, suggested that mineralisation of quinoline by these organisms does not occur, rather, biotransformation to oxygenated analogues of the quinolines are the products. The resulting more polar compounds leads to the increased mobility of the compound in groundwater due to increased solubility and decreased sorption to aquifer material. Methylation (believed to occur after hydroxylation) of these oxygenated analogues, produces metabolites that may persist in the subsurface environments for relatively long periods.

Groundwater isolated from an oil contaminated site in Zealand, Denmark was examined for its aerobic biodegradation potential with quinoline and various other heterocyclic compounds. Indigenous organisms present in the sample rapidly degraded quinoline in under laboratory conditions. (Jensen et al. 1988)

Arvin et al. (1989) observed the biodegradation of quinoline from groundwater (polluted from creosote infiltration) both under lab conditions and in situ in a contaminated aquifer. They modelled the lab reactor on groundwater flow rates and found the in vitro degradation studies mirrored the disappearance of quinoline from the in situ groundwater.

Liu et al. (1994) showed that quinoline could be transformed by organisms in an anoxic freshwater sediment under methanogenic and sulfate reducing conditions, in vitro. Disappearance of quinoline with simultaneous appearance of 2-hydroxyquinoline was observed. In another in vitro experiment, quinoline was added to various surface water samples and concentration of quinoline was monitored over the incubation period. Quinoline was observed to be degraded by indigenous organisms in two eutrophic and an oligotrophic lake waters as well as aeration effluent from a sewage plant, oil refinery and chemical manufacturing plant (Bohonos et al. 1977). Similarly quinoline was added to a river water sample and observed to be degraded in vitro by indigenous organisms present in the river water sample (Cassidy et al. 1988). It was inferred from these in vitro studies of quinoline degradation in environmental water samples, that the biotic potential exists for in situ quinoline degradation.

## 1.2 BACTERIAL QUINOLINE DEGRADATION

Many bacteria have been isolated from a variety of environments (both aerobic and anaerobic) capable of transforming quinoline and its derivatives, a brief survey appears in Table 1-1.

**Table 1-1. Isolated bacteria capable of quinoline degradation.**

| Strain | Pathway | Reference |
|---|---|---|
| Pseudomonas aeruginosa QP | ND | (Aislabie et al. 1990) |
| P. acidovorans CH1 | ND | (Blaschke et al. 1991) |
| P. alcaligenes EL I | ND | (Blaschke et al. 1991) |
| P .chloraphis | ND | (Bennet et al. 1985) |
| P. diminuta 31/1 | ND | (Bott and Lingens 1991) |
| P. fluorescens | ND | (Bennet et al. 1985) |
| P. fluorescens 3 | Coumarin | (Schwarz et al. 1988) |
| P. pseudoalcaligenes | ND | (Bennet et al. 1985) |
| P. pseudoalcaligenes QPS1 | Coumarin | (Shukla 1986) |
| P. putida QP | ND | (Aislabie et al. 1990) |
| P. putida | ND | (Bennet et al. 1985) |
| P. putida (7 strains) | ND | (Schwarz et al. 1988) |
| P. putida 86 | Coumarin | (Schwarz et al. 1989) |

**Table 1-1. cont.**

| Strain | Pathway | Reference |
|---|---|---|
| P. putida QP2 | ND | (Rothenburger and Atlas 1993) |
| P. putida Chin I | ND | (Blaschke et al. 1991) |
| P. testosteroni QPS2 | Aromatic | (Shukla 1987) |
| P. stutzeri QPS4 | Coumarin | (Shukla 1986) |
| P. testosteroni (5 strains) | ND | (Schwarz et al. 1988) |
| Pseudomonas sp 957A | Aromatic | (Brockman et al. 1989) |
| Pseudomonas sp 866A | Aromatic | (Brockman et al. 1989) |
| Pseudomonas sp MPQ | ND | (Aislabie et al. 1990) |
| Pseudomonas sp. CH2 | ND | (Blaschke et al. 1991) |
| Pseudomonas sp. CH3 | ND | (Blaschke et al. 1991) |
| Bacillus circulans 31/2 A1 | ND | (Bott and Lingens 1991) |
| Nocardia sp. QN1 | Aromatic | (Shukla 1987) |
| Moraxella sp. | | (Grant and Al-Najjar 1976) |
| Comamonas acidovorans DSM6426 | Coumarin | (Miethling et al. 1993) |
| Desulfobacterium indolicum DSM 3383 | Coumarin | (Johansen et al. 1997) |
| Rhodococcus spec. B1, F1, F2 | ND | (Schwarz et al. 1988) |
| Rhodococcus spec. B1 | Aromatic | (Schwarz et al. 1989) |
| Rhodococcus sp. Q1 | ND | (O'Loughlin et al. 1996) |
| Rhodococcus sp. CH5 | ND | (Blaschke et al. 1991) |
| Consortium, Pseudomonas (or Xanthomonas) sp Lep1 | Coumarin | (Sutton et al. 1996) |
| Arthrobacter spec. Rü 61 | ND | (Beyer and Lingens 1993) |

In all the cases of quinoline degradation above where intermediates were analysed, 2-hydroxyquinoline was found as the initial product of quinoline degradation, regardless of redox conditions. The following step in the pathway seems to be rate determining as 2-hydroxyquinoline is the only intermediate that has been observed to accumulate.

### 1.2.1 Biochemistry of quinoline metabolism

The biochemistry of bacterial quinoline metabolism has recently been reviewed (Kaiser et al. 1996). Quinoline being composed of two ring structures provides

two main approaches for bacterial catabolism, either the primary attack is on the aromatic or the heterocyclic ring structure. These two possibilities for unsubstituted quinoline catabolism have been observed, each pathway begins with an initial hydroxylation at carbon 2, but then diverge into the opening of the heterocyclic or the aromatic ring.

In this section the tautomeric forms of 2-hydroxyquinoline (2-oxo-1,2-dihydoquinoline) and 2,8-dihydroxyquinoline (8-hydroxy-2-oxo-1,2-dihydroquinoline) are used interchangeably in the text and figure legends to preserve the original authors' nomenclature.

#### *1.2.1.1 Aerobic heterocyclic ring cleavage*

Shukla (1986), working with a *Pseudomonas* sp (QPS-1), isolated from a sewage enrichment culture, found an example of an organism that first attacked the heterocyclic ring. A culture growing on quinoline as a sole source of carbon and nitrogen was monitored and aliquots of the media were analysed at various time intervals for the presence of organic compounds. This study established 2-hydroxyquinoline, 8-hydroxycoumarin, 2,8-dihydroxyquinoline and 2,3-dihydroxyphenylpropionic acid as intermediates of the quinoline degradation pathway with this organism. 2-hydroxyquinoline, 8-hydroxycoumarin and 2,3-dihydroxyphenylpropionic acid could all be metabolised by the quinoline-grown cells. While 2,8-dihydroxyquinoline could be detected in the extracted culture media, when the compound was added to the quinoline grown cells it was only degraded slowly, this observation was accounted for by its low aqueous solubility. In the presence of 5 mM arsenite, when cells were metabolising quinoline, it was found that 2-hydroxyquinoline and 2,3-dihydroxyphenylpropionic acid accumulated in the growth media. The proposed metabolism of quinoline by the *Pseudomonas* sp is illustrated in Figure 1-5. This mechanism of quinoline metabolism, were the pyridine ring is attacked first yielding the intermediates 8-hydroxycoumarin and 2,3-dihydroxyphenylpropionic acid, has been named the "Coumarin" pathway of quinoline degradation.

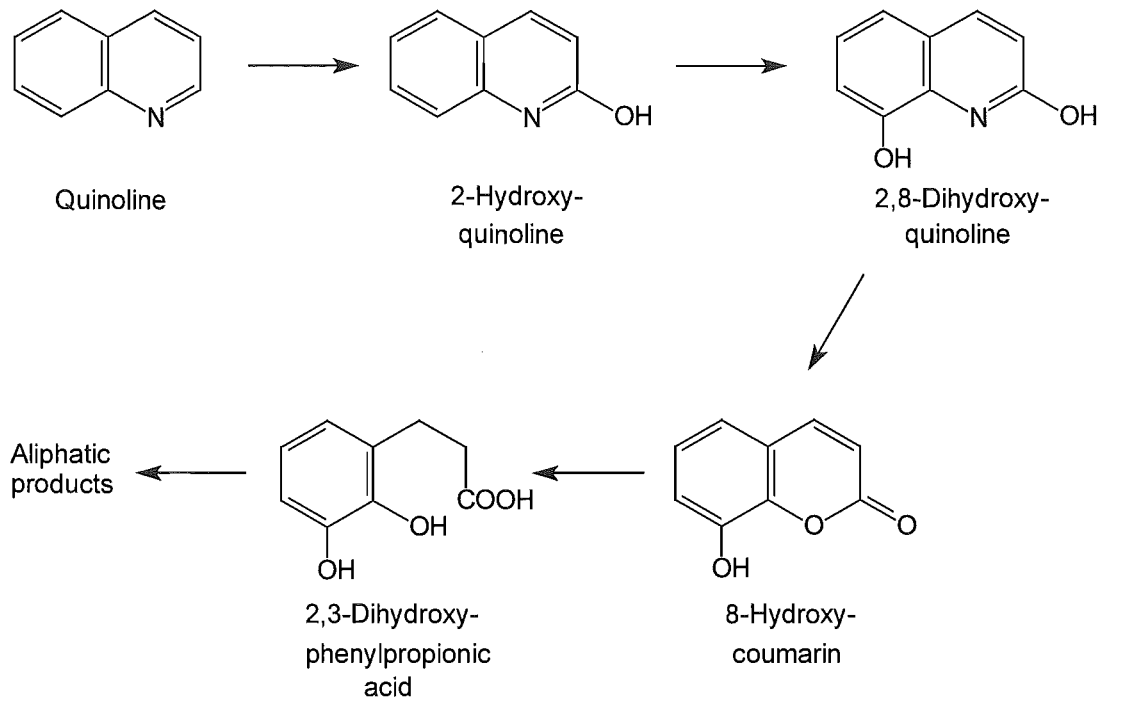


**Figure 1-5. Transformation of quinoline: Pyridine ring cleavage "Coumarin Pathway".** Pathway first described by Shukla (1986) for the *Pseudomonas* sp (QPS-1). NB 2-hydroxyquinoline and 2,8-dihydroxyquinoline spontaneously tautomerise in water (at physiological pH) to 2-oxo-1,2-dihydoquinoline and 8-hydroxy-2-oxo-1,2-dihydroquinoline respectively.

Schwarz et al. (1989) working with a *Pseudomonas fluorescens* and *P. putida* isolates also found 2-oxo-1,2-dihydroquinoline, 8-hydroxy-2-oxo-1,2-dihydroquinoline (spontaneous tautomeric rearrangement of 2-hydroxyquinoline and 2,8-dihydroxyquinoline in water), 8-hydroxycoumarin and 2,3-dihydroxyphenylpropionic acid as intermediates when these two organisms were grown in minimal media amended with quinoline as a sole source of nitrogen and carbon. Again, as with Shukla (1986), these *Pseudomonas* species could not utilise 8-hydroxy-2-oxo-1,2-dihydroquinoline as a substrate. Schwarz et al. (1989) agreed with Shukla (1986) that this may be due to the compounds low aqueous solubility, but further added that the most likely scenario would be that 8-hydroxy-2-oxo-1,2-dihydroquinoline can chelate metal ions, thus making a metal ion complex unavailable to the bacteria in this form. Schwarz et al. (1989) presented a degradation pathway of quinoline for

*Pseudomonas fluorescens* 3 and *P. putida* 86 which was identical to the pathway described for the *Pseudomonas* sp. isolated by Shukla (1986). Schwarz et al. (1989) further proposed a possible mechanism for the removal of nitrogen from the pyridine moiety (Figure 1-6).

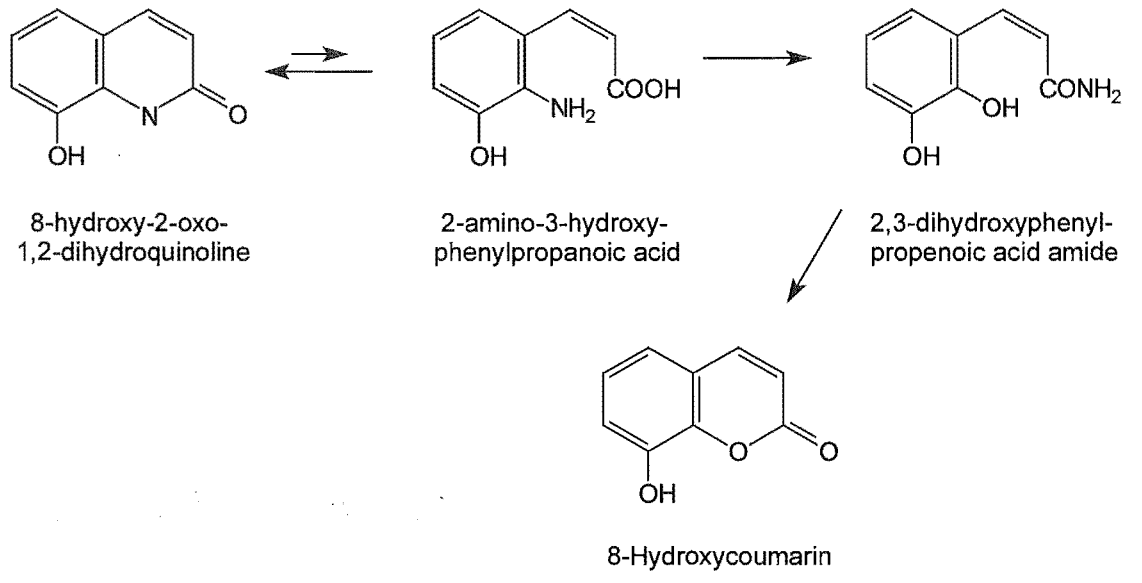


**Figure 1-6. Proposed mechanism of N removal from the pyridine moiety (Schwarz et al. 1989).**

Shukla (1989), when working with a *P. stutzeri* (QPS-4), found this organism degraded quinoline in a similar fashion to other *Pseudomonas* species and identified the same compounds: 2-hydroxyquinoline; 8-hydroxycoumarin and 2,3-dihydroxyphenylpropionic acid as metabolites. 2,8-dihydroxyquinoline was found as a minor metabolite in the growth media, but when supplied to the quinoline grown cells, no significant oxygen uptake by the culture was observed. Shukla (1989) concluded that the chemical and biochemical studies did not support a role for 2,8-dihydroxyquinoline in the conversion of 2-hydroxyquinoline to 8-hydroxycoumarin. The implication from this work is that 2,8-dihydroxyquinoline is a by-product of quinoline metabolism rather than an intermediate in its degradation. Shukla (1989) proposed that the true intermediates between 2-hydroxyquinoline and 8-hydroxycoumarin may be cinnamic acids (Figure 1-7). These open ring structures are unstable and may

only exist bound to enzymes making their isolation impossible under the experimental conditions employed. This would account for the presence of 2,8-dihydroxyquinoline as a product of re-circularisation of (cis)-2-amino-3-hydroxycinnamic acid (Figure 1-7). The sequestering of these ring structures in enzyme complexes would account for their not being detected in the other studies of Shukla (1986) and Schwarz et al. (1989). The suggestion of 2,8-dihydroxyquinoline being a by-product of quinoline metabolism was not addressed in these studies but in both cases this compound could not be metabolised by the examined organisms.

The role of 2,8-dihydroxyquinoline in the metabolism of quinoline and therefore, the catabolism of quinoline via attack of the pyridine ring remains an unresolved issue. Schwarz et al. (1989) include this compound as an intermediate with *Pseudomonas fluorescens* 3 and *P. putida* 86, in their proposed mechanism (Figure 1-6), while Shukla (1989) suggested 2,8-dihydroxyquinoline is a by-product, perhaps as a result of recircularisation of an enzyme bound intermediate, rather than an intermediate itself. Shukla (1989) concluded that further biochemical and enzymatic studies are required to elucidate the details of this pathway, given the significance of the resistance by the pyridine nucleus to electrophillic agents. It is still unknown how the pyridine moiety of the quinoline molecule is cleaved and how nitrogen is eliminated. It is also unknown how 2,3-dihydroxyphenylpropionic acid is further degraded (Schwarz and Lingens 1994).

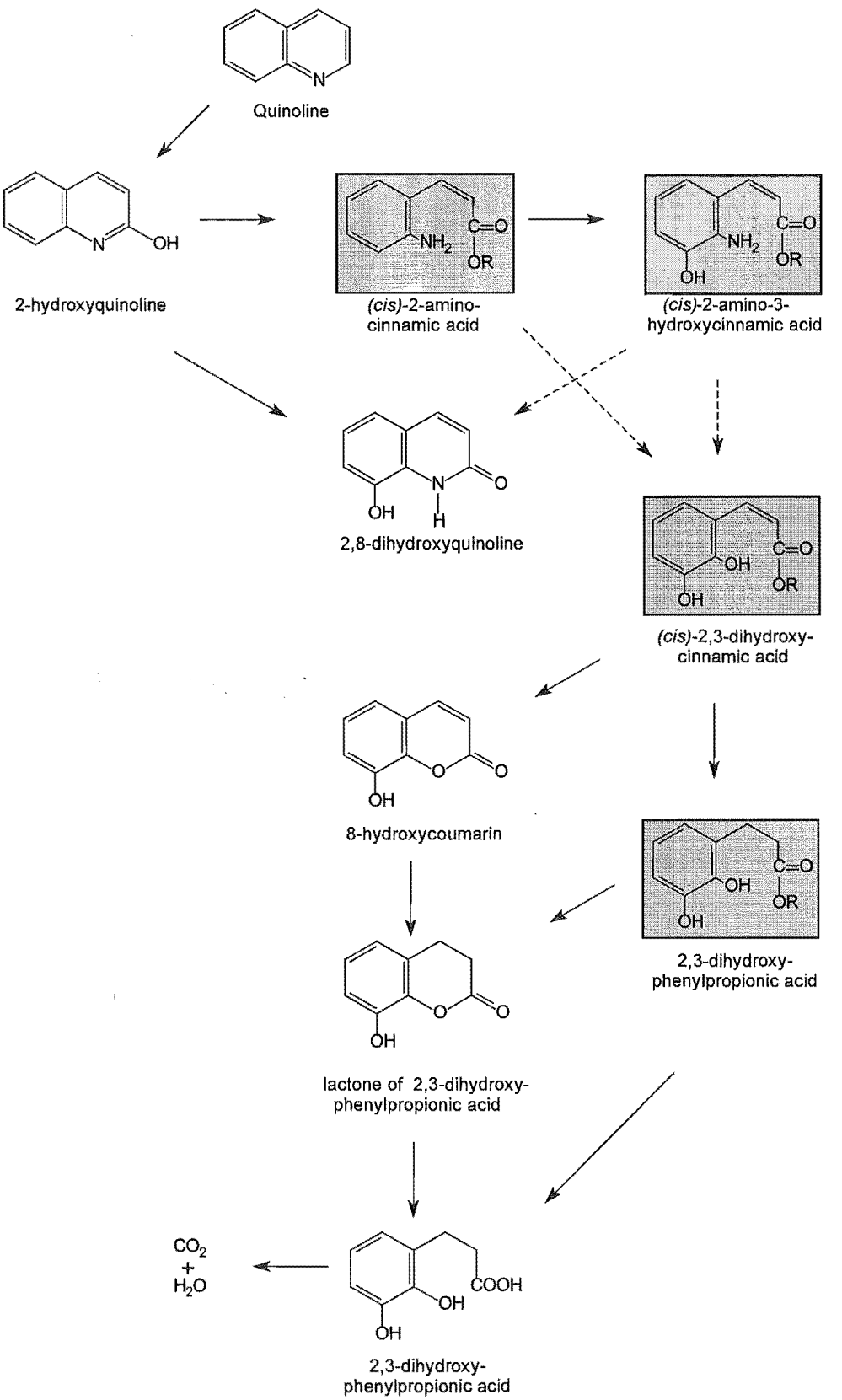


**Figure 1-7. Pathways of quinoline catabolism in *P. stutzeri* (QPS-4) and probable mechanisms for the formation of 8-hydroxycoumarin (Shukla 1989).** Putative enzyme-bound intermediates appear in shaded boxes.

### *1.2.1.2 Aerobic aromatic ring cleavage*

Organisms that degrade quinoline may also begin with the opening of the aromatic (benzene) ring as the primary ring cleavage event. Grant and Al-Najjar (1976) isolated a bacterium from garden soil capable of utilising quinoline as a sole source of carbon and nitrogen. They found the quinoline transformation products 2-hydroxyquinoline, 2,6-dihydroxyquinoline and a trihyrdoxyquinoline (probably 2,5,6-trihydroxyquinoline) as metabolites. This was the first evidence of the "aromatic" quinoline degradation pathway, where the aromatic ring is the first to be cleaved by the bacteria. Another organism metabolising quinoline in a similar fashion to this soil isolate was the *Rhodococcus* B1 isolated by Schwarz et al. (1989), described below (Figure 1-8).

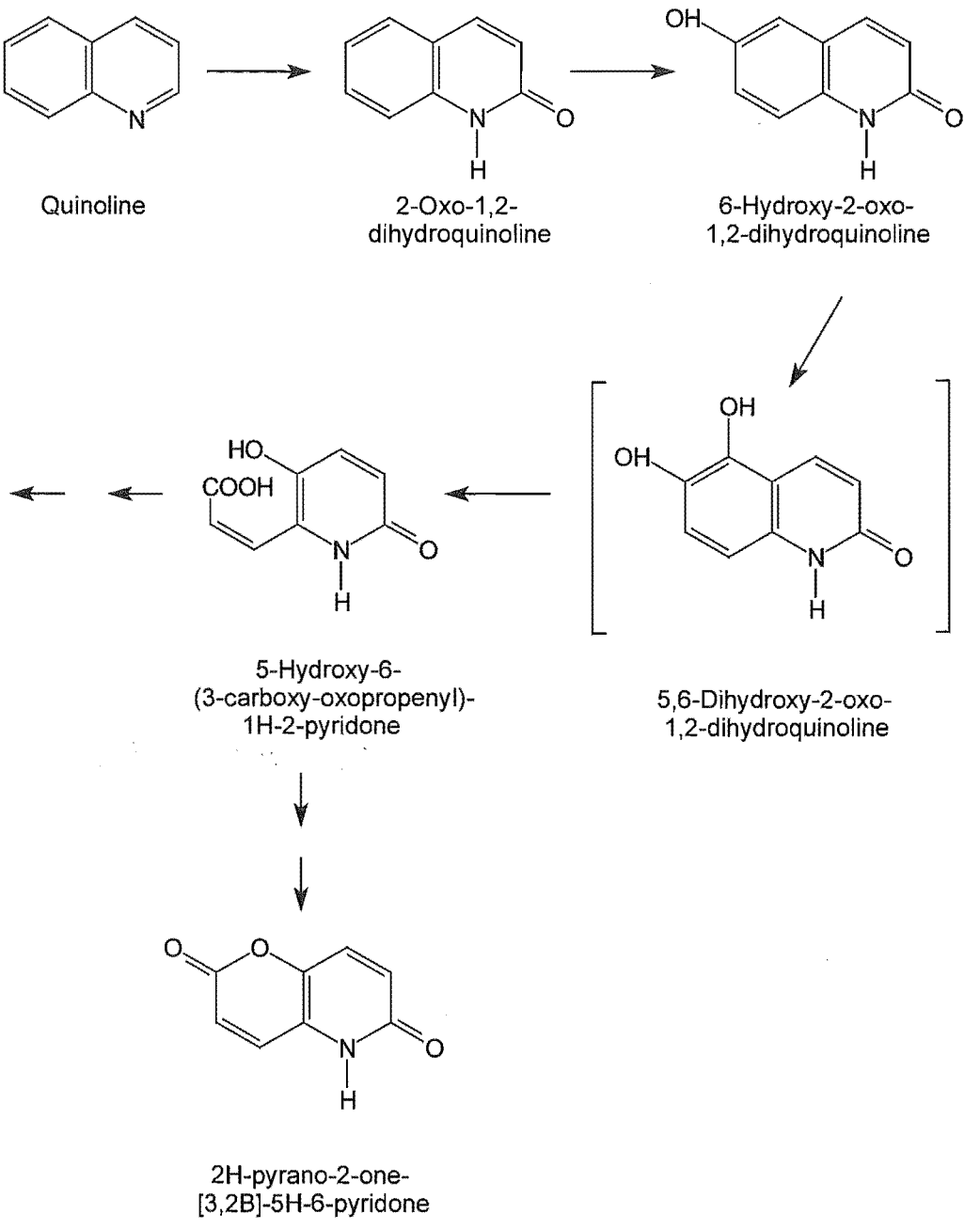


**Figure 1-8. Proposed pathway for the degradation of quinoline by *Rhodococcus spec.* B1 (Schwarz et al. 1989), the "Aromatic pathway".**

As with the pathway first proposed by Shukla (1986), where the pyridine ring is attacked first, conversion of quinoline to 2-oxo-1,2-dihydroquinoline is the first step of the of the degradation of quinoline by this *Rhodococcus* species. Further hydroxylations are then performed on the benzene ring forming the intermediates 6-hydroxy-2-oxo-1,2-dihydroquinoline and 5-hydroxy-6-(3-carboxy-3-oxopropenyl)-1H-2-pyridone. Schwarz et al. (1989) suggest the ring

opening step is proceeded via 5,6-dihydroxy-2-oxo-1,2,dihydroquinoline, this postulated trihydroxyquinoline (seen in brackets in Figure 1-8) was not detected in the culture media, however. 2H-pyrano-2-one-[3,2b]-5H-6-pyridone appears to be a dead-end metabolite that is formed by a nonenzymatic oxidative decarboxylation of 5-hydroxy-6-(3-carboxy-3-oxopropenyl)-1H-2-pyridone, the bacteria are unable to utilise this by-product of the pathway and it accumulates in the growth media. The *meta*-cleavage product, 5-hydroxy-6-(3-carboxy-3-oxopropenyl)-1H-2-pyridone, may be degraded in a similar manner to kynurenic acid catabolism in pseudomonads were the end products are L-glutamate, D- and L-alanine acetate and carbon dioxide (Schwarz et al. 1989).

The first complete pathway for the aromatic degradation of quinoline by a bacteria (*Pseudomonas* strain *866*A) has been proposed (Szecsody et al. 1994) (Figure 1-9). Experimental evidence was not presented for this pathway, however, its derivation is largely inferred from what was known of *meta*-cleavage of the benzene moiety, cleavage of the pyridine ring, quinoline degradation and unpublished observations of *Pseudomonas 866*A.

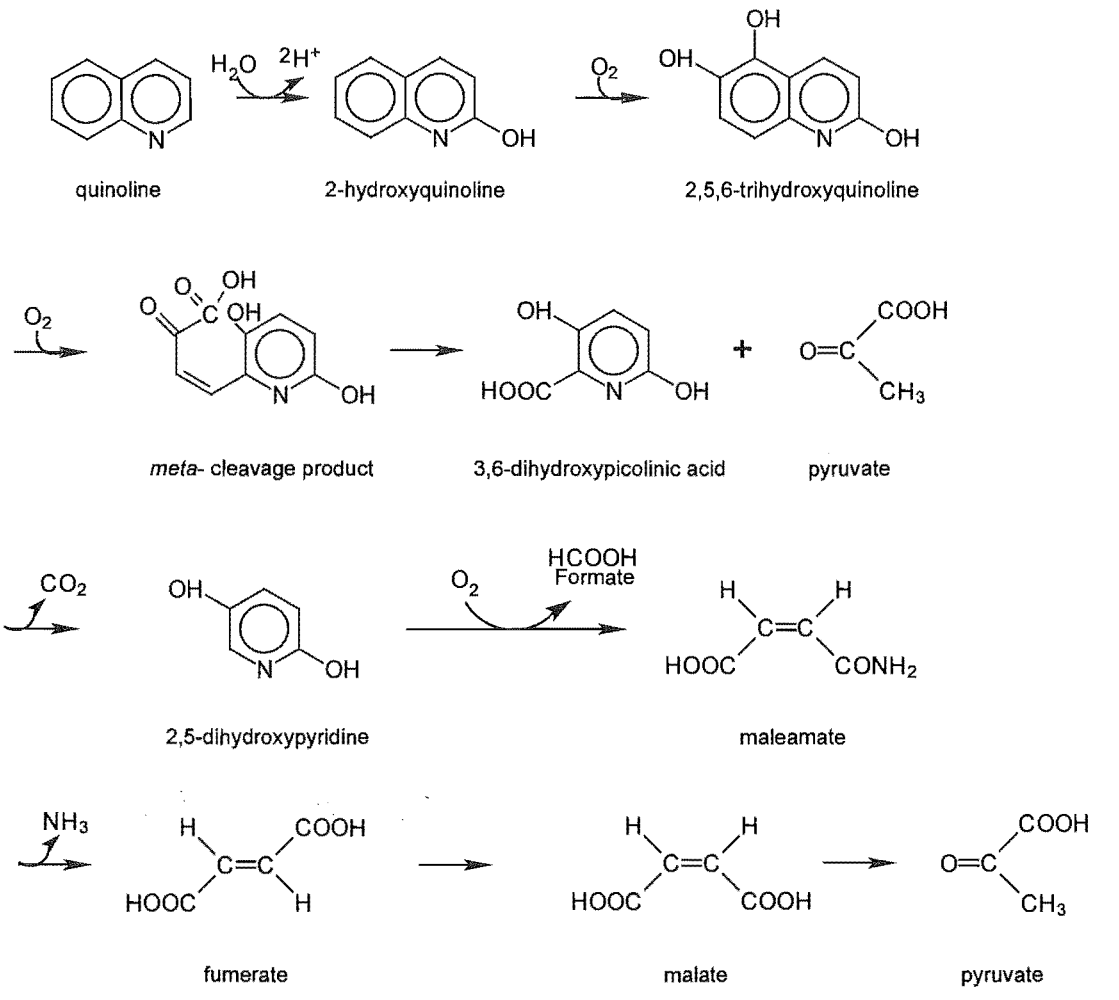


**Figure 1-9. Proposed pathway of quinoline degradation by *Pseudomonas* strain *866A* (Szecsody et al. 1994).**

### *1.2.1.3 Anaerobic quinoline degradation*

Bacterial quinoline degradation has also been shown to occur under anaerobic conditions (Pereira et al. 1987), (Pereira et al. 1988), (Brockman et al. 1989) and (Johansen et al. 1997). Again, as noted with breakdown of quinoline under aerobic conditions, quinoline degradation proceeds via the 2-hydroxyquinoline intermediate. As yet, a single organism capable of complete degradation of quinoline under anaerobic conditions has not been isolated. Partial degradation of quinoline by a single organism and both partial and complete degradation by a consortium of bacteria have been reported.

Anaerobic digester studies where quinoline was added to a methanogenic bacterial consortia isolated from an aquifer contaminated with wood treatment waste, showed no methane production potential of this azaarene when compared to unamended controls. At the conclusion of the experiments GC-MS of extracts of these cultures revealed the presence of 2-(1H)-quinolinone (making up >95% of metabolic by-products) and the methyl derivatives 1-methyl-2 (1H)-quinolinone, 4-methyl-2(1H)-quinolinone and 1,4-dimethyl-2-(1H)-quinolinone (Pereira et al. 1987). Therefore microbial transformation of quinoline had occurred without complete degradation (Figure 1-10). By contrast, complete mineralisation (to methane and carbon dioxide) of the nitrogen heterocycle indole, has been reported under methogenic conditions (Wang et al. 1984).

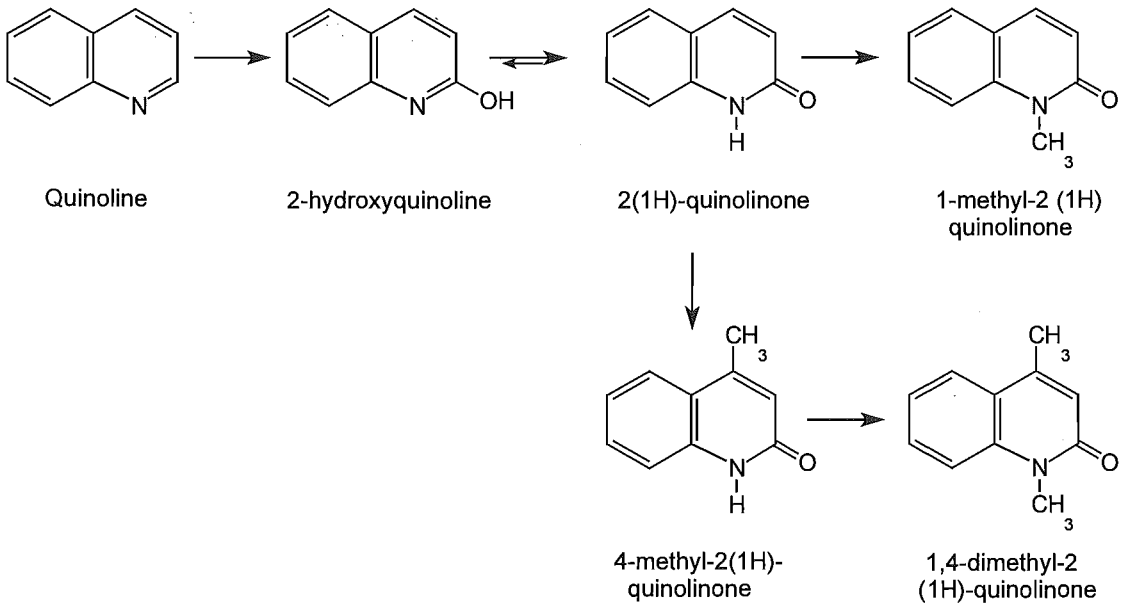


**Figure 1-10. Biotransformation products and proposed quinoline metabolism in static anaerobic cultures.** Adapted from Pereira et al. (1987).

Brockman et al. (1989) examined sediments extracted from a sand aquifer as deep as 203 m below the surface. Under both aerobic and anaerobic conditions, evolution of $^{14}CO_2$ was detected from these sediments when amended with [U-$^{14}$C]quinoline. A pure isolate identified as a *Pseudomonas* sp

(designated 957A) was found able to completely mineralise quinoline under aerobic conditions but could only transform quinoline under anaerobic conditions. Quinoline was found to disappear under anaerobic conditions with a simultaneous production of a soluble metabolite, this metabolite disappeared over the course of the experiment, finally resulting in another apparently non-utilisable soluble metabolite. These anaerobic cultures did not evolve significantly more $^{14}CO_2$ than their poisoned control counterparts, but the authors could not discount that being the case with the original sediment samples. They proposed the possibility existed that a consortium of organisms in this original sediment sample were capable of utilising and mineralising quinoline or the soluble intermediates produced by 957A, thus resulting in the evolution of $^{14}CO_2$.

Johansen et al. (1997) studied the degradation of quinoline under sulphate reducing conditions with *Desulfobacterium indolicum* (DSM3383). It was shown that both quinoline and indole were mineralised by this organism given by the 85% reduction of the total organic carbon in the growth media over the incubation period, and its ability to utilise quinoline as a sole source of carbon and nitrogen It was conceded that mineralisation was probably incomplete, however, as unidentified UV absorbing compounds remained in the media. *D. indolicum* was proposed to degrade quinoline via hydrogenation of the heterocyclic ring (following the apparent universal step of hydroxylation at position two) leading to the detected intermediate 3,4-dihydro-2-quinolinone. As no products with the functional groups RCOOH, ArOH and RCOR were detected in the culture media, it is not known how ring cleavage and nitrogen removal occurs with this organism.

### 1.2.2 Enzymology of quinoline degradation

#### *1.2.2.1 Quinoline oxidoreductase*

One of the first clues for the type of enzymes involved in quinoline metabolism came with the knowledge that water was the oxygen atom donor for the first

step of quinoline metabolism. This conversion of quinoline to 2-hydroxyquinoline (or the lactam 2(1H)quinolinone, the more stable tautomer in aqueous solution at physiological pH) appears to be universal in many studies of bacterial metabolism of unsubstituted quinoline (Grant and Al-Najjar 1976; Shukla 1986; Pereira et al. 1987; Pereira et al. 1988; Schwarz et al. 1989; Johansen et al. 1997). The only examples of exceptions to this observation lie, however, when there is a substitution at position 2 of the quinoline ring. The degradation of 2-methylquinoline (quinaldine), provides an example, were position two is blocked by a methyl group, the hydroxylation is performed at position 4 by *Arthrobacter spec.* Rü 61 (Beyer and Lingens 1993).

To determine where the oxygen atom for the hydroxylation of quinoline was derived from, Pereira et al. (1988) incubated microbial consortia isolated from both aerobic (creosote contaminated soil) and anaerobic (methogenic consortia from sewage sludge) environments in the presence of quinoline and $H_2{}^{18}O$ (and air for the aerobic culture). In both the aerobic and anaerobic cultures incorporation of the $^{18}O$ as an hydroxyl at position 2 of the quinoline ring, without participation of atmospheric oxygen, as demonstrated by mass spectrometry of the extracted 2(1H)quinolinone and high-resolution mass spectrometry of resulting ions.

This first, and seemingly universal step amongst bacteria, of the degradation of quinoline was analysed by Bauder et al. (1990), they suggested that this reaction was catalysed by an enzyme they named quinoline oxidoreductase (Figure 1-11).

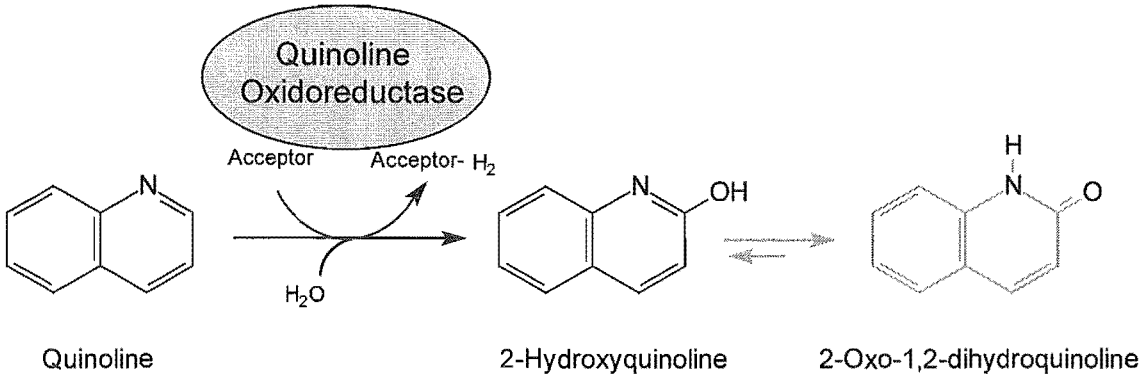


**Figure 1-11. Reaction catalysed by quinoline oxidoreductase.** Adapted from Bauder et al. (1990). Reaction in light grey is spontaneous tautomeric rearrangement and is not enzyme mediated.

Bauder et al. (1990) suggested that the reaction was analogous to other reactions catalysed by molybdenum containing enzymes such as nicotinic acid dehydrogenase, nicotine dehydrogenase and xanthine dehydrogenase which also catalyse an oxidation at position 2 of a nitrogen containing heterocyclic ring.

Initial growth experiments with *P. putida* 86 showed that this organism required molybdenum for growth on quinoline and if tungstate (a molybdenum antagonist), was added, growth on quinoline was abolished. Growth of this organism on 2-oxo-1,2-dihydroquinoline was independent of molybdenum supplementation. These results suggested that molybdenum was required for the first oxidation of quinoline but subsequent reactions did not require this trace element. They also verified the oxygen atom in this oxidation reaction came from water using $H_2{}^{18}O$, mass spectrometry of the products revealed the incorporation of $^{18}O$ into 2-oxo-1,2-dihydroquinoline. At the same time, Blaschke et al. (1991) noted that hydroxylation of heterocyclic compounds beside the heteroatom are often performed by molybdenum-containing dehydrogenases. This group, working with several *Pseudomonas* species and a *Rhodococcus* sp., also found that tungstate caused a marked inhibition of growth for these organisms while using quinoline as a substrate and concluded that quinoline degradation must include a molybdenum dependent reaction.

Partially purified enzyme extracts from *P. putida* Chin IK were isolated, spectral and fluorescence data were typical of those expected for a molybdenum-containing dehydrogenase with flavin and iron sulphur centers present. It was found that the enzyme had an $M_r$ of 300 kDa in the *Rhodococcus* sp and in all the several *Pseudomonas* species analysed. SDS-PAGE of the protein isolated from *P. Putida* Chin IK revealed 3 bands: 85; 30 and 18 kDa. This enzyme was found to be inhibited by cyanide and arsenite but not methanol. Immuno-cross reactions were tested for this enzyme and two other molybdenum dehydrogenases: nicotinate dehydrogenase (*Bacillus niacini*) and xanthine dehydrogenase (*P. putida* Fu1); no cross reaction occurred with these two enzymes and quinoline dehydrogenase was observed

Quinoline oxidoreductase isolated from *P. putida* 86 was purified to homogeneity (Bauder et al. 1990) and some of its physical and enzymatic properties were examined. It was found that the native protein's molecular mass was 300 kDa. With polyacrylamide gradient gel electrophoresis under denaturing conditions, 3 protein bands were seen, corresponding to $M_r$ 85 kDa, 30 kDa and 20 kDa, integration of these protein bands suggested the native molecule was made of two of each of these subunits. The absorption spectrum of quinoline oxidoreductase was revealed to similar to nicotine dehydrogenase, milk xanthine oxidase and chicken and turkey liver xanthine dehydrogenase. The cofactor composition of this enzyme was found to be similar to other molybdo-iron/sulphur-flavoproteins with a Mo:Fe:S:FAD ratio of 2:8:8:2. Again, like other molybdoenzymes, quinoline oxidoreductase was inhibited by methanol and cyanide. The substrate specificity of quinoline oxidoreductase was narrow with only quinoline, 5-hydroxyquinoline, 6-hydroxyquinoline, 7-hydroxyquinoline, 8-hydroxyquinoline and 8-chloroquinoline being accepted as substrates. With the modified quinolines the enzyme activity ranged from 5 to 28 % compared to quinoline. The physiological electron acceptor for this enzyme was not found, in these experiments artificial electron acceptors were employed.

The prosthetic group of quinoline oxidoreductase (isolated from *P. putida* 86) was further analysed and found to exist as a molybdopterin cytosine dinucleotide (Hettrich et al. 1991), this variation of the molybdopterin has only been observed in carbon monoxide dehydrogenase isolated from *Pseudomonas carboxydoflava*. This was in contrast to the xanthine dehydrogenase isolated from *P. putida* 86 which contained only the molybdopterin, with no cytosine dinucleotide (Hettrich et al. 1991). Hettrich and Lingens (1991) studied the xanthine dehydrogenase isolated from *P. putida* 86 and found marked physical differences in this enzyme and the quinoline oxidoreductase isolated from the same bacterium. These results indicate that two different molybdenum pterin cofactors exist within the same organism (Hettrich et al. 1991). The quinoline oxidoreductase isolated from *P. putida* 86 was further analysed by electron paramagnetic resonance spectroscopy, this technique detected four redox active centres and revealed two non-identical iron-sulphur clusters in the enzyme (Tshisuaka et al. 1993).

Peschke and Lingens (1991) isolated a quinoline oxidoreductase from *Rhodococcus spec.* B1 and purified the enzyme to homogeneity. This enzyme has many similar characteristics to the quinoline oxidoreductases isolated from *P. putida* 86, above. The molecular mass of the native protein was 300 kDa, under denaturing and reducing conditions 3 protein bands were revealed at 82, 32 and 18 kDa. As with *P. putida* 86, 2 of each of these proteins make up the subunits of the enzyme. The cofactor of this enzyme was revealed to be identical to that of *P. putida* 86 with a Mo:Fe:S:FAD ratio of 2:8:8:2 (Peschke and Lingens 1991), in addition, the molybdopterin exists as a cytosine dinucleotide in both organisms (Hettrich et al. 1991). Protein N terminal sequence analysis of each of the three subunits were also found to be identical to those from *P. putida* 86, at that time, however, no other similarities with published sequences were found. The *Rhodococcus* quinoline oxidoreductase has a broader substrate range, in addition to the hydroxyquinoline derivatives, the methyl derivatives: 3-methylquinoline, 4-methylquinoline and 8-methylquinoline could also be oxidised but only at about 20% the rate of

quinoline. Peschke and Lingens (1991) concluded that this enzyme is very similar to quinoline oxidoreductase found in *P. putida* 86 but there are minor differences in isoelectric point and molecular mass of the subunits of this *Rhodococcus* enzyme. It was concluded that the quinoline 2-oxidoreductases from distantly related organisms exhibit far-reaching similarities (Rosche et al. 1995).

Quinaldine oxidoreductase has been isolated from by *Arthrobacter spec.* Rü 61 (Beyer and Lingens 1993). This enzyme catalyses the addition of a hydroxyl group at position 4 of the heterocycle, as position 2 is occupied by a methyl group. This enzyme has many similar properties to the quinoline oxidoreductases described above including a molybdopterin cytosine dinucleotide cofactor. Quinaldine oxidoreductase will also catalyse the conversion of quinoline to 1*H*-4-oxoquinoline. It is not known how these hydroxylases, which catalyse their substrates in an apparently analogous manner, selectively mediate the addition of hydroxyl groups on different positions of the heterocyclic ring.

#### *1.2.2.2 2-oxo-1,2-dihydroquinoline 8 monooxygenase*

The second enzyme in the coumarin pathway of quinoline degradation, 2-oxo-1,2-dihydroquinoline 8 monooxygenase, was isolated from *P. putida* 86 (Rosche et al. 1995). The enzyme was purified from cell extracts and found to be a two component system, comprising of a 37 kDa reductase and 330 kDa oxidase component. The reductase component was found to be a monomer containing one flavin adenine dinucleotide and one plant-type ferredoxin (2Fe-2S) cluster. NADH donates 2 electrons simultaneously as a hydride to this reductase component, the iron sulphur centres are restricted to one electron transfers.

The function of the reductase was determined to mediate the two electron/one electron transfer from the hydride by its flavin cofactor. The oxygenase consists of 6 identical subunits of 55 kDa each containing a single Rieske-type

iron-sulphur cluster (2Fe-S). The oxygenase is reduced by the reductase enabling the hydroxylation of the substrate. Ferrous iron is also required for catalytic function and was proposed to be a weakly associated cofactor of this oxygenase component. The net result being that the reductase transfers 2 electrons from NADH to the oxygenase component of the enzyme, where in the presence of molecular oxygen, a hydroxylation reaction at position 8 on the substrate, 2-oxo-1,2-dihydroquinoline, occurs (Figure 1-12).

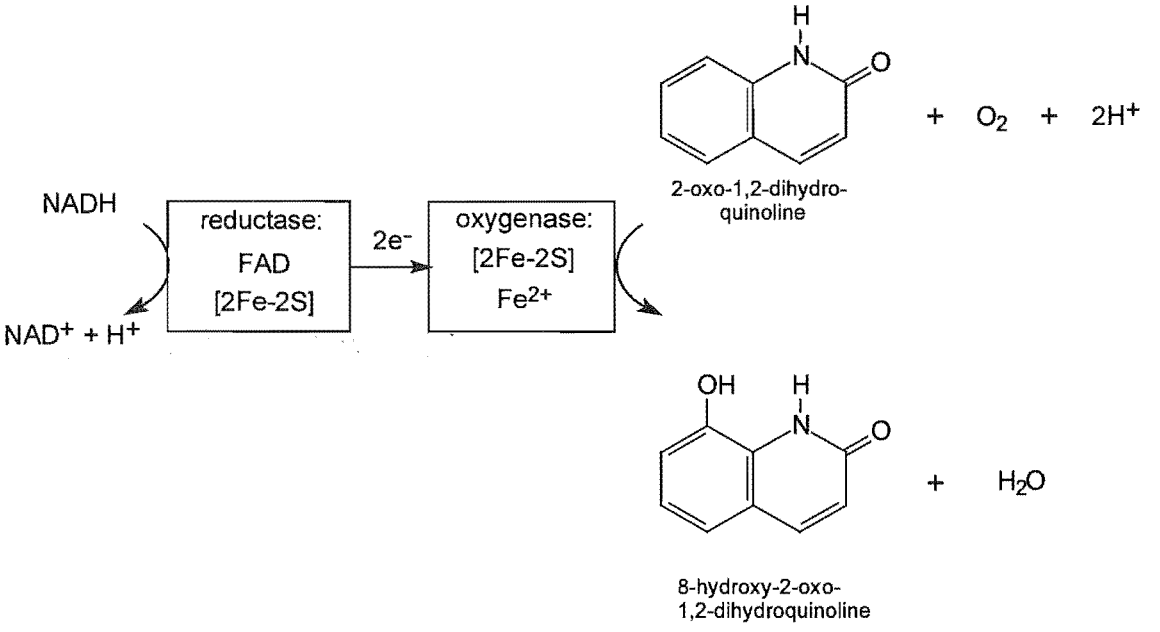


**Figure 1-12. Proposed catalytic mechanism of 2-oxo-1,2-dihydroquinoline 8-monooxygenase (Rosche et al. 1995).**

Determination of $O_2$ uptake showed a 1:1:1 stoichiometry for 2-oxo-1,2-dihydroquinoline:$O_2$:NADH, $O_2$ was an absolute requirement, no reaction was detected under anaerobic conditions. Substrate specificity was found to be high, with 25 substrates (of a related nature) tested none were converted, 8-hydroxyquinoline, 8-hydroxy-2-oxo-1,2-dihydroquinoline and coumarin showed substrate dependent oxygen consumption without being converted. The explanation for this observation was suggested to be caused by uncoupling of the electron transfer from substrate hydroxylation with concomitant production of hydrogen peroxide.

It was conceded by Rosche et al. (1995) that their results did not entirely discount the possibility that the enzyme is a dioxygenase instead of a monooxygenase. 8-hydroxy-2-oxo-1,2-dihydroquinoline may be a product of spontaneous dehydration of an unstable 7,8- or 8,8a-dihydrodiol of 2-oxo-1,2-dihydroquinoline, however, no dihydrodiol could be detected in any *in vitro* experiment.

Rosche et al. (1995) suggest that 2-oxo-1,2-dihydroquinoline 8-monoxygenase belongs in group IB of the multicomponent non-heme iron oxygenases. Class I enzymes are two component with a flavin (FAD in class B) and a plant -type ferredoxin (2Fe-2S) in the reductase. This group also includes 2-halobenzoate 1,2-dioxygenase and benzoate 1,2-dioxygenase, these two enzymes are heteromultimers, in contrast to the homomultimer configuration of 2-oxo-1,2-dihydroquinoline 8-monoxygenase. The monomultimer configuration is seen with the IA class of the multicomponent non-heme iron oxygenases, this group however, use FMN as the flavin cofactor.

### 1.2.3 Genetics of quinoline metabolism

The genes encoding the quinoline 2-oxidoreductase (*qorS*, *qorM* and *qorL*) and 2-oxo-1,2-dihydroquinoline 8-monoxygenase (*oxoO* and *oxoR*) from *P. putida* 86 have been cloned and DNA sequence analysis performed (Bläse et al. 1996 & Rosche et al. 1997).

#### *1.2.3.1 qor genes (Bläse et al. 1996)*

A library of total genomic DNA isolated from *P. putida* 86 was collected in a cosmid vector and maintained in an *E. coli* background. This library was probed with oligonucleotides designed from the six amino acids in the N-terminal region of the large subunit (QorL) of quinoline 2-oxidoreductase. Four of the *E. coli* containing cosmid clones returned positive signals, but no catalytic activity could be detected in the *E. coli* hosts. Three explanations were offered

for the lack of quinoline 2-oxidoreductase activity in *E. coli*. *Pseudomonas* promoters are poorly recognised by *E. coli* RNA polymerases due to little similarity to the consensus sequences of *E. coli* promoters. The G+C content of *Pseudomonas* at 67-68% compared to 50% of *E. coli* which may affect codon-anticodon interaction in the *E. coli* host. If *qor* gene expression were to occur in *E. coli*, formation of an inactive Qor protein might result if *E. coli* could not synthesise the cytosine dinucleotide required for the molybdenum pterin cofactor. *E. coli* have been found to exclusively contain the guanine dinucleotide form of the molybdenum pterin cofactor

The four cosmids were isolated from their *E. coli* host and used to transform a non-quinoline metabolising *P. putida* strain. One of these transformant clones was capable of cometabolising quinoline in the presence of succinate. Further experiments revealed that accumulation of 2-oxo-1,2-dihydroquinoline was only transient and was metabolised by the *P. putida* transformant. Indeed, when grown on minimal media with quinoline as a sole source of carbon, nitrogen and energy, the transformed *P. putida* was capable of growth. This result indicated that this cosmid, which contained a 30 kb insert, has the genes which encode for all the other enzymes of the "coumarin pathway" of quinoline degradation and these enzymes are produced in a catalytically active form enabling this *P. putida* to mineralise quinoline.

This cosmid (pCIB119), which allowed *P. putida* to grow on quinoline, was digested with *Eco*RI, an 11.5 kb fragment from this cosmid was found to hybridise to degenerate primers deduced from the N-terminal region of both the large subunit (QorL) and the small subunit (QorS) of quinoline 2-oxidoreductase. From this 11.5 kb *Eco*RI fragment, 4585 bp of DNA sequence data was generated. Three open reading frames were identified in this region and were designated *qorS, qorM* and *qorL* corresponding to the QorS (small), QorM (medium) and QorL (large) protein subunits of quinoline 2-oxidoreductase. The open reading frames were arranged in the transcriptional order (5')-*qorM-qorS-qorL*-(3') (Figure 1-13), no further open reading frames

were detected upstream of *qorM* or downstream of *qorL* in the 4585 bp fragment. This transcriptional order is identical to all other described structural genes of molybdenum-containing prokaryotic hydroxylases with the LMS or $L_2M_2S_2$ configuration.

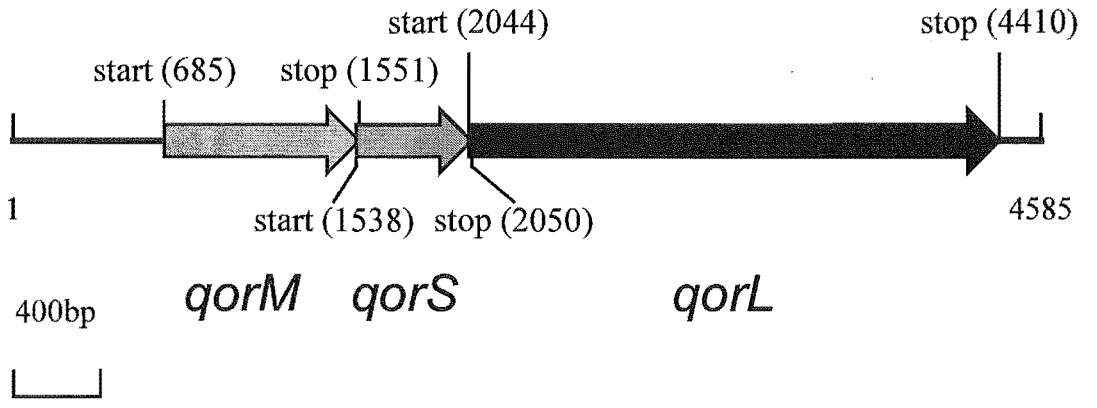


**Figure 1-13. Arrangement of the *qor* genes of *P. putida* 86.** Compiled from Bläse et al. (1996).

The arrangement of the *qor* genes can be seen in Figure 1-13, the region begins with a ribosomal binding site (RBS) at position 671 and ends with the stop codon at 4410. *qorM* is 867 bp in length which corresponds to a protein of 288 aa long with a molecular mass of 30 650 Da. The potential RBS (AAGTAGGTGA) is located 14 bp upstream of the start codon at position 671. *qorS* coding region is 513 bp and overlaps the terminus of *qorM* by 14 bp (Figure 1-13). The potential RBS (AAGGAGCT) begins at position 1526, 12 bp upstream of the start codon. The translated protein is 168 aa long corresponding to a mass of 18 012 Da. The coding region for the large subunit, *qorL*, is 2367 bp long, this corresponds to a 788 aa translated peptide of mass 84 113 Da. A putative RBS (AGGAG) is located at position 2032. Therefore, the molecular mass of putative peptides translated from *qorM, qorS* and *qorL* correspond very closely to the SDS-PAGE analysis of QorM, QorS and QorL (Bauder et al. 1990) being 30 kDa, 20 kDa and 85 kDa respectively.

The G+C content of this *qor* region was found to be 62.82%, which matches the published G+C content of 62.5% reported for *P. putida* biovar A.

The translated protein sequences of the 3 genes were compared to other (nine eukaryotic xanthine dehydrogenases and five prokaryotic hydroxylases) molybdenum-containing hydroxylases possessing a monooxo-monosulphido-type molybdenum center.

QorS was found to show a 25% sequence similarity (identical or physiochemically related amino acids) to the compared protein sequences. Two distinct motifs were revealed by this sequence analysis involving conserved cysteines. The first cysteines was presumed to correspond to a binding site of bacterial and plant-type [2Fe-2S] ferredoxins with the consensus motif C-$X_4$-C-G-X-C-$X_n$-C. n=11 for prokaryotic molybdenum-containing hydroxylases (including Qor), n=21 in all described eukaryotic xanthine dehydrogenases and n=29 in most bacterial and plant ferredoxins. The second set of 4 cysteines in the QorS sequence correspond to the motif C-G-X-C-$X_{31}$-C-X-C, this sequence is conserved in all described sequences. These two cysteine containing motifs were assumed to be the binding sites of the two [2Fe-2S] centers which had been observed by electron paramagnetic resonance spectroscopy of the enzyme in Tshisuaka et al. (1993).

QorM showed the least similarity of the three subunits to the other compared sequences at 11.1% identical or similar amino acids. QorM was suspected to contain the FAD binding domain as with other molybdenum containing hydroxylases, however, no consensus of known FAD binding sequence could be found in QorM. There was some conservation of glycines between these sequences which have been shown to be involved in FAD binding. The area of FAD binding in prokaryotic molybdenum-containing hydroxylases is still unknown, making difficult to make any conclusions of FAD binding regions in amino acid sequences.

QorL shares a 12.3% similarity between the sequences studied. This large subunit is expected to contain the molybdenum molybdopterin cytosine dinucleotide (MMCD) and substrate binding site. The crystal structure of aldehyde oxidoreductase (MOP) from *Desulfovibrio gigas* had been recently determined (Romão et al. 1995). The enzyme is composed of two subunits each with two different [2Fe2S] centers and a MMCD. One of these subunits showed a 42% amino acid sequence similarity to QorL. Within MOP three molybdopterin binding segments and two dinucleotide binding segments were discovered. These five amino acid sequences show some conservation in all the 14 organisms studied including Qor from *P. putida* 86. The authors plan to investigate the essential amino acids in these 5 sequences using site directed mutagenesis to aid the understanding of the binding of the MMCD. Studies to elucidate substrate binding at the active site and the mode of FAD binding were also suggested.

#### *1.2.3.2 oxoO & oxoR genes (Rosche et al. 1997)*

The genes encoding 2-oxo-1,2-dihydroquinoline 8-monoxygenase, as with the *qor* genes, were also isolated from the cosmid pCIB119. Oligonucleotide probes prepared from both the oxidative and reductive subunits of the enzyme revealed these components were encoded separately and localised either side of the *qor* genes (Figure 1-14).

Both *oxoO* and *oxoR* have ATG translational start codons preceded by a putative ribosomal binding sequence 5'-GGAG-3'. The protein encoded by *oxoO* is 446 amino acids long with a calculated mass of 51.2 kDa. *oxoR* encodes a protein of 343 amino acids with a calculated mass of 37 kDa. The G+C content of *oxoO* and *oxoR* is 61.6 and 63.2% respectively. Codon usage for both these genes showed preferential usage of G and C in the third position.

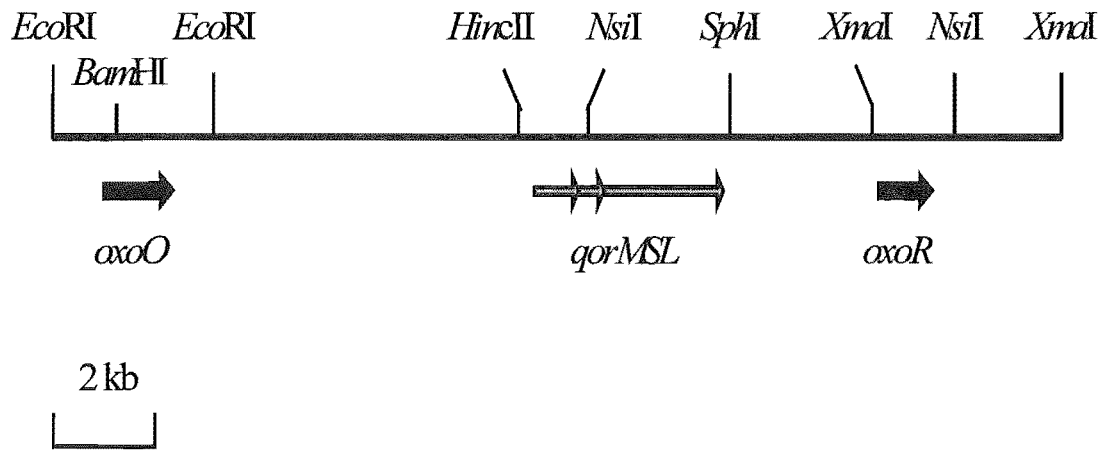


**Figure 1-14. Restriction map showing orientation of *oxoR* and *oxoO* in relation to the *qorMSL* genes.** From Rosche et al. (1997).

Comparisons of amino acid sequences of OxoR and OxoO to other known reductases and oxygenases characterised 2-oxo-1,2-dihydroquinoline 8-monoxygenase as being composed of a class IA-like oxygenase and a class IB-like reductase. The evolutionary divergence between these two classes and the unusually large distance (15 kb) between the *oxoO* and *oxoR* genes led the authors to speculate that the genes were of a separate origin and have been combined to produce a novel enzyme.

## 1.3 *PSEUDOMONAS AERUGINOSA* QP

*P. aeruginosa* QP was isolated from an enrichment culture composed of phosphate salts medium with 1.2 mM quinoline as a sole source of carbon, an oil contaminated soil served as an inoculum (Aislabie et al. 1990). The pure isolate was identified as *Pseudomonas aeruginosa* using Rapid NFT strips (Analytab Products), growth at 42 °C and production of a fluorescent green pigment were also seen as salient features. Quinoline was degraded by this organism under aerobic conditions only, to a maximum concentration of 12 mM. Quinoline degradation was analysed under laboratory conditions, $10^7$ cells were used to inoculate phosphate salts medium amended with 2.5 mM quinoline, typically no detectable aromatic compounds remained in the medium after 24 hr

incubation at 28 °C with aeration. Incubation of *P. aeruginosa* QP with quinoline resulted in accumulation of 2-hydroxyquinoline in the growth media, this was first detected after 9 hr incubation and peaked after 14-15 hr, by 17 hr this intermediate product had disappeared.

The substrate range of *P. aeruginosa* QP was examined and the organism was found able to hydroxylate 6-methylquinoline, 7-methylquinoline and 8-methylquinoline, no further degradation of these methylated compounds took place, even in the presence of succinate and an alternate nitrogen source. The same methylated quinoline derivatives were also hydroxylated by *P. aeruginosa* QP when supplied in an oil shale, quinoline and 2 other unidentified compounds were also degraded from this complex mixture. The quinoline relatives, isoquinoline, pyridine and indole were also tested as substrates, with and without alternative nitrogen and carbon sources, no degradation of these compounds by *P. aeruginosa* QP was observed (Aislabie et al. 1990).

The plasmid profile of *P. aeruginosa* QP was analysed using the method of Eckhardt (1978) and the organism was found to be a host to four plasmids of 320, 250, 225 and 180 kb pairs in size. Plasmid curing experiments, mediated by serial transfers of media containing SDS, were performed, it was concluded from these experiments that the 250 and 225 kb plasmids were essential for the complete degradation of quinoline by this organism. Presence of the 320 kb plasmid, with either (or neither) of the 250 and 225 kb plasmids, resulted in accumulation of the 2-hydroxyquinoline intermediate only. *P. aeruginosa* Q, a strain which was cured of all four plasmids, could not degrade quinoline to any degree. These results suggest that the gene(s) required for hydroxylation of quinoline must exist in (at least) duplicate both on the 320 kb plasmid and on at least one of the 220 or 250 kb plasmids. The 180 kb was not essential to quinoline degradation as its presence or absence did not affect the strains' ability to degrade quinoline (Aislabie et al. 1990). The plasmid curing experiments, however, did not rule out the possibility that the plasmid might

contain duplicate copies of genes from the other larger plasmids. However, plasmid isolation attempts in a later study (Horridge 1991) failed to reveal the presence of plasmids in QP.

## 1.4 AIMS OF THIS STUDY

*P. aeruginosa* QP has been shown to degrade quinoline (Aislabie et al. 1990), it was not known by which (if either) of the described pathways of quinoline metabolism this organism utilises. Transposon mutagenesis of QP will provide mutants with various phenotypes unable to degrade quinoline. Mutants with varying abilities to degrade quinoline would accumulate intermediates of the degradation pathway and the genes specifying the various steps of the pathway would be labelled with transposon insertions. When this study began, nothing was known of the bacterial genetics of quinoline metabolism. The specific objectives of this study are listed below.

1) Produce an agar plate assay for the rapid screening of the quinoline degrading phenotype.

2) Ascertain whether QP harbours plasmids and determine if this DNA is associated with quinoline degradation.

3) Perform transposon mutagenesis on QP, characterise the mutant phenotypes of quinoline degrading deficient mutants. Analyse for the presence of quinoline degradation intermediates and compare to previously described intermediates of the coumarin and aromatic pathways of quinoline degradation.

4) Clone the DNA surrounding transposon insertion and produce restriction maps of these insertions.

5) Clone the representative wild-type DNA of transposon inserted DNA and use in complementation assays of quinoline degrading deficient mutants.

6) Test any plasmids which complement quinoline degrading deficient mutants for expression of gene products.

7) DNA sequence analysis of cloned QP DNA and compare to known DNA sequences stored at databases.

# 2. MATERIALS AND METHODS

## 2.1 BACTERIAL STRAINS AND PLASMIDS

Bacterial strains and plasmids used in this study are listed in Table 2-1.

**Table 2-1. Bacterial strains and plasmids.**

| Strain or plasmid | Genotype or description | Source or Reference |
|---|---|---|
| ***E. coli*** | | |
| AB1157 | thr-1 leuB6 ara-1 his-4 argE3 thi-1 proA2 xyl-5 mtl-1 lacY1, galK2, rpsL31 tsx33(t6R) supE44 | Lab Stock |
| DH5α | supE44 ΔlacU169(ϕlacZΔM15) hsdR17 thi-1 relA1 recA1 endA1 gyrA96 | (Hanahan 1983) |
| S17-1 | recA294::Tn7 ($Tp^R$, $Sm^R$) $tra^+$ (contains chromosomally integrated RP4 derivative) | (Simon et al. 1983) |
| ***P. aeruginosa*** | | |
| QP | Quinoline degrading prototroph | (Aislabie et al. 1990) |
| QP100 | QP, $Rf^R$ | (Horridge 1991) |
| QP101 | QP, $Sm^R$ | (Horridge 1991) |
| QP100-1 | QP100, contains Tn5-B21 insertion, slow quinoline degrader | This study |
| QP100-2 | QP100, contains Tn5-B21 insertion, unspecified auxotroph | This study |
| QP100-3 | QP100, contains Tn5-B21 insertion, unspecified auxotroph | This study |
| QP100-4 | QP100, contains Tn5-B21 insertion, unspecified auxotroph | This study |
| QP100-5 | QP100, contains Tn5-B21 insertion, unspecified auxotroph | This study |
| QP100-6 | QP100, contains Tn5-B21 insertion, unable to grow on quinoline ($Qln^-$) | This study |
| QP100-7 | QP100, contains Tn5-B21 insertion, unable to grow on quinoline ($Qln^-$) | This study |

**Table 2-1. cont.**

| Strain or plasmid | Genotype or description | Source or Reference |
|---|---|---|
| ***P. aeruginosa*** cont. | | |
| QP100-8 | QP100, contains Tn5-B21 insertion, unable to grow on quinoline ($Qln^-$) | This study |
| QP100-9 | QP100, contains Tn5-B21 insertion, slow quinoline degrader | This study |
| OT2 | *leu*-1 | Lab stock |
| OT11 | *leu*-1 *pro*-1 $Sm^R$ | This study |
| OT109 | *ilv*B112 *leu*-1 *pro*-5 $Sm^R$ (pRO1614) | This study |
| OT684 | *leu*-1 *lys*-1 *res*-4 $Rf^R$ | (Potter and Loutit 1982) |
| OT899 | *ilv*B112 *leu*-1 *pro*-5 $Sm^R$ (RP4) | Lab stock |
| OT906 | clinical isolate, prototroph | Dunedin Hospital |
| ***Agrobacterium tumefaciens* C58** | Contains 192 kb Ti plasmid and >300 kb cryptic plasmid | Barry Scott, Massey University |
| **Plasmids** | | |
| pACYC184 | $Cm^R$ $Tc^R$ | Lab stock |
| pBR322 | $Ap^R$ $Tc^R$ | This study |
| pUC19 | $Ap^R$ | This study |
| pRO1614 | $Ap^R$ $Tc^R$ | (Olsen et al. 1982) |
| pSUP102 | Mobilisable pACYC184 derivative, Tn*5*-B21 vector | (Simon et al. 1989) |
| pNHQ8 | pACYC184 containing the 17 kb *Bgl*II fragment from QP100-8 | This study |
| pNHQ81 | pUC19 containing the 9 kb *Bam*HI fragment from pNHQ8 | This study |
| pNHQ82 | pUC19 containing the 1.7 kb *Bam*HI/*Hin*dIII fragment from pNHQ8 | This study |
| pNHQ83 | pUC19 containing the 0.5 kb *Bam*HI/*Eco*RI fragment from pNHQ8 | This study |
| pNHQ84 | pUC19 containing the 3 kb *Eco*RI fragment from pNHQ8 | This study |
| pC1 (pG5, pG6) | pUC19 containing the 5 kb *Bam*HI fragment from QP100 | This study |

**Table 2-1. cont.**

| Strain or plasmid | Genotype or description | Source or Reference |
|---|---|---|
| **Plasmids** cont. | | |
| pNHQR2 (pNHQR1 & pNHQR3) | pRO1614 containing the 4.5 kb BamHI/EcoRI fragment from pC1 | This study |
| pNHQR4 | pNHQR2 with the 3 kb EcoRI fragment from pNHQ84 | This study |
| pNHQR8 | pNHQR2 with 3 kb EcoRI fragment from pNHQ84 (opposite orientation to pNHQR4) | This study |

## 2.2 BUFFERS AND MEDIA

Buffers, solutions and specific reagents used in this study were prepared as described in Appendix II. Media used in this study were prepared as described in Appendix I.

### 2.2.1 Antibiotics and media supplements

Antibiotics were added to agar plates or to liquid media as stated in Table 2-2. Resistance to ampicillin, for example, was designated $Ap^R$ or sensitivity; $Ap^S$.

**Table 2-2. Antibiotics.**

| Antibiotic | Abbreviation | *E. coli* concentration | *P. aeruginosa* concentration |
|---|---|---|---|
| Ampicillin | Ap | 100 μg/ml | N/A |
| Carbenicillin | Cb | N/A | 500 μg/ml |
| Chloramphenicol | Cm | 20 μg/ml | N/A |
| Gentamycin | Gm | 20 μg/ml | 200 μg/ml |
| Kanamycin | Km | 50 μg/ml | 500 μg/ml |
| Rifampicin | Rf | 100 μg/ml | 200 μg/ml |
| Streptomycin | Sm | 50 μg/ml | 500 μg/ml |
| Tetracycline | Tc | 25 μg/ml | 250 μg/ml |

Antibiotics were stored as 1000× stock solutions at -20 °C. Tetracycline and rifampicin were dissolved in methanol. All other antibiotics were dissolved in $dH_2O$ and filter sterilised through 0.22 μm pore Millipore filters.

Where X-Gal was required on agar plates, 40 μl of a solution of X-Gal (20 mg/ml in dimethylformamide) was spread on to the surface of the plate along with 4 μl of IPTG (200 mg/ml in sterile $dH_2O$), the plate was allowed to dry for 30 min at 37 °C before inoculation, as described in Sambrook et al. (1989). Proline and leucine were added to minimal media at 2.0 and 0.3 mM when required. When supplementary nitrogen was needed, it was supplied as $NH_4NO_3$ at 1g/l.

Quinoline (Aldrich) was added to liquid media at 0.03% (v/v) (approximately 2.5 mM), for agar plates, unless otherwise stated, quinoline was added at 0.03% (v/v) but first emulsified in Tween 80 (Sigma) and $dH_2O$ as described in 2.4.3 below.

## 2.3 BACTERIOLOGICAL METHODS

### 2.3.1 Culture conditions

Both *E. coli* and *P. aeruginosa* strains were incubated at 37 °C, unless otherwise stated. For liquid cultures, single colony inoculations were made into 10 ml volumes of LB contained in Universal bottles, these were incubated o/n with shaking (250 rpm) in a gyrotory water bath (New Brunswick, G76D). When required, the cells were collected by centrifugation (6000*g*, 2 min). Media were solidified with 1.5% (w/v) agar (Difco) when required. These agar plates were inoculated and incubated o/n.

### 2.3.2 Storage of bacterial cultures

For the long term storage of bacteria, single colony inoculations were made into LB medium and grown under appropriate conditions o/n then concentrated 2

fold by centrifugation and amended with glycerol, 15% (v/v) for *P. aeruginosa* and 40% (v/v) for *E. coli* strains, before being stored at -80 °C. For working stocks of cultures, inoculum from the frozen stocks were streaked onto LB agar plates (amended with appropriate antibiotics, when necessary) and incubated at 37 °C o/n. These plates could be stored at 4 °C for up to 2 weeks, single colony inoculations were made from these cultures during that time.

## 2.4 QUINOLINE UTILISATION ASSAYS

### 2.4.1 Effect of liquid media composition on the rate of quinoline degradation by QP

Media of 3 different compositions: medium A; medium B; and medium C (Appendix I for composition of these media) were evaluated for their ability to support *P. aeruginosa* QP while growing on quinoline as a sole source of carbon, nitrogen and energy. The aim of these experiments was to find the medium which supported the fastest degradation of quinoline by QP cells. Medium A, a phosphate salts medium, was used by Shukla (1986) for support of a *Pseudomonas* sp. while mineralising quinoline. Medium B described by Schwarz et al. (1988) was used to support a variety of bacteria growing on quinoline including *P. putida*. Medium C was a combination of Bushnell-Haas medium (without added nitrogen) and the trace element-salts mixture used by Schwarz et al. (1988) in their medium composition.

Into 250 ml volume conical flasks, a 100 ml volume of each medium amended with 0.03% (v/v) quinoline was placed in triplicate, each flask was inoculated with a 0.1 ml aliquot of a stationary phase culture of *P. aeruginosa* QP previously grown in LB. For each medium, a cell free control flask containing quinoline was also set up. The flasks were incubated at 30 °C with shaking (200 rpm) and 0.1 ml samples were taken periodically and subject to UV spectrum analysis to monitor quinoline disappearance.

The molybdenum requirement of QP, while growing on quinoline, was evaluated as follows. QBHS medium (without added molybdenum) was inoculated with QP cells, which had previously been washed (three times) in the same medium to reduce the risk of carry-over molybdenum contamination, in 4 separate flasks in triplicate. Each of the sets of flasks were amended with 0.1 μM tungstate, molybdate or a combination and one set of flasks without either supplement. Turbidity ($A_{600}$) of the cultures were scored after a 36 hr incubation at 30 °C with aeration.

### 2.4.2 UV spectrophotometry of quinoline growth media

The disappearance of quinoline from liquid culture could be observed by UV spectrophotometry. Quinoline has a characteristic UV absorption spectrum, deviations from this spectrum suggest a reduction in the concentration of quinoline and/or its modification.

Media was harvested from growth flasks, bacterial cells and precipitated matter were removed by centrifugation at 12000*g* for 2 min. A 100 μl of this supernatant was diluted into one ml of $dH_2O$ and transferred to quartz crystal cuvettes (1 cm light path). The spectra of the diluted supernatants were then analysed from 200 to 350 nm on a diode array spectrophotometer (Hewlett Packard 8452A).

### 2.4.3 Plate assay

Loss of ability to metabolise quinoline as a sole source of carbon, nitrogen and energy was evaluated by screening for colonies unable to grow on appropriate agar plates. These assay plates were composed of QBHS medium solidified with 1.5% (w/v) purified agar (BRL) and amended with quinoline. As quinoline did not dissolve readily in the culture media, to ensure homogeneity three delivery techniques of quinoline to the media were investigated: application in ether; vapour and an emulsification method.

1) Application in ether 12 µl of quinoline was dissolved per ml of diethyl ether. This quinoline solution could then be added to the plate either by spraying several applications and allowing the ether to evaporate before each new application. Alternatively adding 0.5 ml of the quinoline solution directly to the plate's surface, with swirling to ensure even distribution. Ether was allowed to evaporate before inoculation of the agar plate.

2) Vapour method 10 µl of quinoline was applied to a sterile filter paper attached to the inside of the petrie dish lid. The agar plate was inoculated and sealed with plastic wrap, the bacteria were allowed to grow in the presence of quinoline vapour in the headspace of the dish.

3) Emulsification method A stock quinoline emulsion was made by vortexing 0.6 ml quinoline, 0.4 ml Tween 80 and 4 ml of sterile $dH_2O$. Volumes (120 µl of this stock provides 30 µl of quinoline, enough for 100 ml of media) of this emulsion were added, with mixing, to molten agar prior to pouring the agar plates.

In each case, the assay plates were inoculated by transferring the bacterial colonies to be tested using a sterile toothpick. Gently stabbing the assay plate agar's surface, forming a shallow depression, ensured a good transfer of the inoculum and a visual indication of the point of inoculation. Each plate was inoculated with a positive (QP) and a negative (non quinoline degrading *P. aeruginosa*) control. The inoculated plates were sealed with cling-wrap to prevent the volatile quinoline escaping and incubated at 30 °C.

## 2.5 BACTERIAL CONJUGATION CONDITIONS

### 2.5.1 Matings in liquid media

Recipient strains were incubated o/n in LB broth with shaking (200 rpm) at 37 °C, they were diluted 1:1 with fresh medium and returned to the incubator 30

min before mixing with donor cells. Donor strains were incubated for four hours under identical conditions, with antibiotic selection for the plasmid, where appropriate. Donor cells were first washed with fresh LB to remove antibiotics, then mixed with recipients in a 1:10 ratio. Matings were performed in a Universal bottle containing a 5 ml volume of LB at 37 °C for 2 hours. Following this incubation period, the bottles containing the mating mixture were briefly vortexed, aliquots were then spread on LB agar plates containing appropriate selective agents.

### 2.5.2 Filter matings for transposon mutagenesis

Recipient strains were incubated o/n in BHN (brain-heart broth, Difco, amended with 4g/L $KNO_3$) medium with shaking (200 rpm) at 37 °C. Donor strains were incubated under identical conditions but for four hours and with appropriate antibiotic selection. Donor cells were centrifuged and washed with fresh medium, the cells were re-centrifuged then resuspended in the original volume of LB. Donor and recipient cells (0.75 ml of each) were mixed in a microfuge tube and collected by centrifugation. The supernatant was aspirated and the cell pellet was resuspended in 50 μl of fresh BHN medium. The cell suspension was spread onto a 0.45 μm pore nitrocellulose filter (Millipore) previously placed on an BHN agar plate. Cells were mated in this fashion at 37 °C for 4-24 hours.

### 2.5.3 Filter matings for transposon mutagenesis (QP100 grown at 43 °C)

A loopful of the recipient QP100 cells was inoculated into 10 ml of BHN and grown o/n at 43 °C (without aeration). One hundred microlitres of this culture was used to inoculate a second 10 ml aliquot of BHN, the cells were allowed to grow at 43 °C for 18 hr, without aeration. The donor strain, *E. coli* S17-1 (pSUP102-Gm Cm::Tn*5*-B21) was grown o/n, 100 μl of this culture was used to inoculate 10 ml of fresh LB/Tc medium, the cells were grown at 37 °C with vigorous shaking (300 rpm) for 4 hours. An 800 μl aliquot of these donor cells were washed in fresh LB, re-centrifuged before being mixed with an equal volume of the QP100 recipient cells, which had previously been incubated at 50

°C for 4 min. This mating mix was sedimented by centrifugation and the cell pellet was resuspended in 50 μl of BHN before being spread on a Millipore filter (0.45 μm pore) placed on a BHN agar plate. Following a 12 hour incubation at 37 °C, the cells were scraped off the filter and resuspended in 1 ml of BHN, 100 μl aliquots of this suspension were spread onto Rf/Tc LB agar plates and incubated o/n.

## 2.6 DNA MANIPULATION AND CLONING TECHNIQUES

### 2.6.1 Ethanol precipitation of DNA

DNA in aqueous solutions was precipitated, unless otherwise stated, by addition of 1/10 volume of 3M sodium acetate (pH 5.2) and 2 volumes of ethanol. Following an incubation (typically 30 min) at -20 °C, the DNA was collected by centrifugation (12000*g* at 4 °C for 10 min in an Eppendorf 5403 centrifuge) and the supernatant was removed by aspiration. The DNA pellet was washed with 70 % (v/v) ethanol/$dH_2O$, and re-centrifuged (1 min), the resulting DNA pellet was aspirated and dissolved in an appropriate volume of $dH_2O$ or TE buffer and stored at -20 or 4 °C.

### 2.6.2 Quantification of DNA in aqueous solution

The DNA to be measured was dissolved in 1 ml of TE buffer and transferred to a quartz crystal cuvette (1 cm light path) and the UV absorption spectrum of the solution was measured on a diode array spectrophotometer (Hewlett Packard 8452A). The ratio of the absorbency at 260nm/280nm was calculated, if the value was 1.8 (±0.1) then the concentration of the dsDNA was assumed to be 50 μg/ml for every absorbance unit at 260nm (Sambrook et al. 1989).

### 2.6.3 Phenol:Chloroform extractions

Contaminating proteins were removed from DNA solutions with phenol:chloroform and chloroform extractions according to Sambrook et al. (1989). This protocol was used where DNA purity from plasmid minipreparations was inadequate or following enzymic modification of DNA to remove and inactivate the added enzymes.

An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the DNA sample and vortexed until a homogeneous emulsion formed. The organic and aqueous phases were separated by centrifugation at 12000*g* for 2 min. The aqueous phase was removed and placed in a fresh tube. This step was repeated once. The aqueous phase was then extracted as above but with an equal volume of chloroform, the DNA was recovered by ethanol precipitation.

### 2.6.4 Alkaline-lysis minipreparation of plasmid DNA

Standard plasmid minipreparations were performed as detailed in Sambrook et al. (1989) with minor modifications.

Typically 1.5 ml aliquots of an o/n culture, grown under appropriate selection for the plasmid of interest, were transferred to microfuge tubes and centrifuged. The resulting cell pellet was resuspended in 100 μl of solution 1 with vigorous vortexing. The mixture was placed on ice and 200 μl of solution 2 was added with gentle inversion to effect mixing of the resulting viscous solution. Following a 5 min incubation on ice, 150 μl of solution 3 was added with gentle mixing to form a flocculant white precipitate. Following a further 15 min incubation on ice, the microfuge tube was centrifuged at 15000*g* for 10 mins at 4 °C to collect the precipitate. The supernatant was decanted into a fresh microfuge tube, 250 μl of ice-cold isopropanol was added, the tube was incubated at -20 °C for approximately 30 min to precipitate the DNA. The microfuge tube was then centrifuged, as before, and the supernatant discarded. The collected DNA

pellet was washed with ice-cold 70% ethanol and briefly (1 minute) re-centrifuged, the DNA pellet was redissolved in 200 µl of 0.3 M sodium acetate (pH 5.2). The DNA was re-precipitated by addition of 400 µl of ice cold ethanol and incubation for 30 min at -20 °C. Finally, plasmid DNA was collected by centrifugation and redissolved in 30 µl of TE buffer.

### 2.6.5 Lysis by boiling plasmid DNA extraction

This method was adapted (J Klena pers. comm.) from the "lysis by boiling" plasmid preparation which appears in Sambrook et al. (1989). This was the method of choice for pUC19 based recombinant plasmids with small inserts.

Typically, 1.5 ml aliquots of an o/n culture, grown under appropriate antibiotic selection, were centrifuged at 4000*g* for 2 min. After the resulting cell pellet was gently resuspended in 300 µl of STET buffer solution, 27 µl of the lysozyme solution was added with gentle mixing. The microfuge tube was placed in a boiling water bath for 45 sec then allowed to cool to room temperature before centrifugation at 15000*g* for 5 min. The resulting white gelatinous precipitate was removed with a sterile toothpick and 328 µl of isopropanol (-20 °C) was added to the supernatant. The DNA was collected by centrifugation at 15000*g*, 4 °C for 15 min, washed with 70% ethanol, re-centrifuged (1 minute) and finally dissolved in 25 µl of TE buffer.

### 2.6.6 Large-plasmid minipreparation

This technique was used for rapid, large plasmid analysis of *P. aeruginosa* strains. It is a modification of a technique from Kieser (1985).

Fresh bacterial cells were produced by inoculating 10 ml volumes of LB, in Universal bottles, with 100 µl aliquots of o/n cultures and incubating at 37 °C at 300 rpm for 4 hours. Bacterial cells were harvested from 1.5 ml volumes in a microfuge (Hereaus BiofugeA) at full speed for 2 min. The cells were re-suspended in 500 µl of lysis solution and incubated at room temperature for 20

min. Lysis was achieved by addition of 250 µl of the alkaline/SDS mix with gentle inversion and incubation at 55 °C for 20 min. After tubes had cooled to room temperature, 250 µl of the acid-phenol solution was added and mixed by gentle flicking of the tube. To achieve homogeneity in this viscous solution, several repetitions of withdrawal and expulsion of the solution through a cut off 1 ml pipette tip (to increase aperture diameter to approximately 3 mm to minimise shearing forces on large DNA molecules) attached to a micropipette was performed until a milky-yellow homogenous solution resulted. The organic and aqueous phases were separated by centrifugation in the microfuge (12000 rpm) for 2 min, typically 30 µl of the upper aqueous phase mixed with 10 µl of loading buffer were analysed by AGE.

### 2.6.7 Large scale plasmid extraction with CsCl purification

This method was employed for the large scale preparation of pure plasmid vector DNA and is adapted from Sambrook et al. (1989). Centrifuge steps were performed in a Heraeus Varifuge 20RS centrifuge and the in a Beckman L8.M Ultracentrifuge.

A single bacterial colony was used to inoculate a 10 ml volume of LB, with appropriate antibiotic selection, and incubated o/n. This culture was then used to inoculate 1 L of LB in a 2 L conical flask, the culture was incubated at 37 °C with vigorous shaking (≈300 rpm) for approximately 18 hours. If, however, the plasmid to be prepared had a relaxed mode of replication, then the culture was incubated until $A_{660}$=0.6 when 170 mg of chloramphenicol was added to culture, the incubation was then allowed to continue for the remainder of the 18 hours.

The cells were harvested by centrifugation. The cell pellet was resuspended in 36 ml of lysis buffer (solution 1), 4 ml of lysis buffer containing 40 mg/ml lysozyme was then added with gentle mixing. Following a 10 min incubation at room temperature, 80 ml of solution 2 was added with gentle swirling. The mixture was incubated on ice for 10 min, after which, 40 ml of ice-cold 5 M potassium acetate (solution 3) was added with thorough mixing. This mixture

was incubated on ice for a further 30 min, it was then centrifuged at 8000 rpm for 10 min at 4 °C (Sorvall GSA rotor) to remove the white precipitate. The resulting supernatant was strained through a stainless steel mesh (0.8 mm mesh size) and a 0.6 volume of ice-cold isopropanol was added. This mixture was incubated at -20 °C for 30 min to precipitate the DNA which was collected by centrifugation at 8000 rpm (GSA rotor). The resulting pellet was dissolved in 16 ml of TE (10 mM Tris-HCl, 10 mM EDTA; pH 8.0). This nucleic acid solution was neutralised by the addition of an appropriate volume of 2 M Tris-HCl (pH 7.0).

The plasmid DNA was now ready for purification by CsCl density gradient centrifugation. For visualisation of the DNA in the gradient, 3 ml of ethidium bromide (5 mg/ml) was added. For each millilitre of this solution, 1 g of CsCl was dissolved with gentle agitation. Precipitated matter was then removed by centrifugation at 10000 rpm for 10 min (Sorval SS-34) then the supernatant was transferred to Beckman polyallomer tubes. These samples were then centrifuged at 40000 rpm (Beckman Ti 75) for 40 hours at 10 °C. The contents of the tubes were viewed under longwave UV light (Mineralight® UVSL.25 set at "longwave"), two fluorescent bands were visualised, the lower, brighter plasmid DNA band was removed with a hypodermic syringe and 18 gauge needle. The ethidium bromide was removed from plasmid DNA by adding an equal volume of CsCl-saturated isopropanol and inverting the tube several times. The isopropanol layer was removed, and the process was repeated until no pink colour was present in the isopropanol or aqueous layers, usually about 7 iterations. The resulting solution was dialysed (12 kDa pore size tubing) against 2× 2 L of TE at 4 °C with gentle stirring over an 18 hour period to remove CsCl. The DNA was further purified by precipitation in 1/10 vol 3M sodium acetate (pH 5.2) and 2.5 vol cold ethanol, incubated at -20 °C for 30 min and then centrifuged at 12000 rpm for 10 min at 4 °C (Sorvall SS-34). Finally the plasmid DNA pellet was dissolved in TE, the concentration recorded, and stored in aliquots at -20 °C.

### 2.6.8 Total genomic DNA preparation

Genomic DNA was prepared from *P. aeruginosa* strains using a modification of the method described by Owen and Borman (1987).

*P. aeruginosa* strains were grown o/n at 37 °C, with shaking, in 10 ml volumes of LB with selection, when necessary. A 1 ml volume of cells were sedimented in a microfuge and resuspended in 1 ml of SE buffer. Following centrifugation, the SE buffer was replaced with 480 µl of SET buffer, the cells were resuspended and brought to 1% (w/v) SDS by addition of 20 µl of 25% (w/v) SDS stock solution. Following a 20 min incubation at 37 °C, proteinase K was added to a total of 50 µg/ml and the microfuge tubes were then returned to the incubator for a further 30 min. These lysates were extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform. To the aqueous supernatant, one half of a volume of 2.5 mM ammonium acetate was added, then a 0.54 volume of ice-cold isopropanol, the microfuge tubes were then incubated at -20 °C for 30 mins. The DNA was collected by centrifugation (12000*g*, 4 °C, 15 min), the pellet washed twice with 70% ethanol and the resulting pellet was dired briefly at room temperature. The DNA was redissolved in 30 µl TE buffer and its concentration and purity recorded.

### 2.6.9 Restriction endonuclease digestion of DNA

Restriction digestions were carried out using the manufacturers' recommended conditions and buffers. Typically reactions were carried out in 20 µl volumes and incubated from 1 to 4 hours. For double enzyme digestions of DNA, the most suitable buffer for the 2 enzymes was used. In some cases it was not possible to choose a buffer to suit all the enzymes involved. In these situations the DNA was digested with each endonuclease separately, ethanol precipitation of the DNA was performed between each digestion.

### 2.6.10 Agarose gel electrophoresis

Agarose gel electrophoresis (AGE) for the separation of DNA molecules was performed as follows. Agarose (0.6-1.2% w/v, depending on fragment size) was dissolved in either 1×TAE or 1×TBE buffer (electrophoresis was performed in the same buffer). DNA samples were mixed with loading buffer before being loaded into the prepared gel. Electrophoresis was accomplished using the Hoefer mini horizontal Submarine Unit gel system. When a larger format was required, typically when greater resolution was required, a Bio-Rad gel mould of 140x128mm was used. Agarose gels were stained with a solution of ethidium bromide (0.5 μg/ml w/v) for 20 minutes and de-stained in $dH_2O$ for 10 minutes, if required. The DNA was visualised using a UV transilluminator (Ultraviolet products Ltd. Tm15). Results were photographed using a Polaroid MP4 Land camera with iso 3000 Polaroid black and white instant film. Migration of fragment sizes could be compared to known standards and with the aid of the computer program Dnafrag version 3.03 (National Research Council of Canada), the size of the DNA fragments could be calculated.

### 2.6.11 DNA extraction from agarose- "phenol/melt" protocol

Specific DNA fragments which had been previously separated by AGE could be extracted from low-melting-temperature agarose (Low Melt Preparative Grade, Bio-Rad) using this protocol.

DNA was treated with restriction endonucleases and separated by AGE where standard agarose had been replaced with low-melting-temperature agarose. Following staining with ethidium bromide, the DNA was visualised with a handheld UV lamp (Mineralight® UVSL.25) set at "longwave", the appropriate DNA fragments were excised from the agarose gel with the minimum volume of agarose. This agarose slice was transferred to a microfuge tube containing 5 volumes of TE (20 mM Tris-HCl, 1 mM EDTA, pH 8.0) and was incubated at 65 °C for 5 minutes to melt the agarose. An equal volume of phenol (pH 8.0) was added, vortexed to form a milky precipitate, then the aqueous layer containing

the DNA was separated by centrifugation at 4000*g* for 10 min. The aqueous phase was extracted with phenol:chloroform, then chloroform alone. The DNA was recovered by addition of a 0.2 volume of 10 mM ammonium acetate and 2 volumes of ethanol and collected by centrifugation at 12000*g* for 10 min at 4°C. The DNA pellet was dissolved in TE or $dH_2O$.

### 2.6.12 GeneClean® purification of DNA

DNA could be extracted from agarose or purified from aqueous solution, before further enzyme treatments, using the GeneClean® III kit manufactured by BIO 101 *Inc.*

When a specific DNA fragment was required from a mixture of different sized fragments, the following protocol was performed. DNA fragments were first separated by AGE, following staining with ethidium bromide, the DNA was visualised with a handheld UV lamp (Mineralight® UVSL.25) set at "longwave". The appropriate DNA band was excised with the minimum volume of agarose and placed in a microfuge tube with 3 volumes of the NaI solution. This mixture was incubated at 50 °C for 5 min to dissolve the agarose. A 5 µl aliquot of EZ-Glassmilk® was added to this DNA solution and incubated at room temperature for 5 minutes, the contents of the tube were periodically agitated gently to prevent settling. The Glassmilk® -DNA complex was retrieved by centrifugation (12000 rpm, 5 s) and resuspended in 500 µl of the New Wash solution before being centrifuged, as before. The pellet was washed three times in this manner. The DNA was liberated from the Glassmilk® by re-suspension in $dH_2O$ or TE buffer and the Glassmilk® was removed by centrifugation.

Alternatively, if DNA was to be removed from an aqueous solution, such as separation from a restriction enzyme, for example, the above protocol was followed with some minor modifications. Three volumes of the NaI reagent were added to the DNA solution with 5 µl of the Glassrnilk®. Following the 5 minute incubation as described above, the Glassmilk® was washed with the

New Wash reagent (2-3 times), and the DNA was liberated and stored in an identical manner to that described above.

### 2.6.13 Ligation of DNA with cohesive ends

Ligation of DNA with overlapping ends was performed in 20 µl volumes according to the enzyme manufacturers' (Gibco BRL) instructions. Typically the vector to insert ratio was 1:3 with up to 200 ng of DNA per reaction. The DNA was dissolved in 15 µl of $dH_2O$, incubated at 42 °C for 5 min to melt any annealed cohesive ends, then allowed to cool before 4 µl of 5× ligation buffer and 1 µl of T4 DNA ligase was added. The reactions were incubated o/n at 14-16 °C. Aliquots of 2-5 µl could be used directly for electroporation, or the DNA was purified with GeneClean® prior to introduction to bacteria.

### 2.6.14 Dephosphorylation of 5' DNA

When ligating a plasmid vector to an insert sequence of DNA where both the ends of the DNA were cut with the same enzyme, the cleaved vector DNA was treated with HK™ Phosphatase (Epicentre Technologies) to preclude vector self ligation. If the vector DNA to be dephosphorylated had been cut with a heat-labile restriction enzyme then following heat inactivation, the buffer was brought to a total of 5mM $CaCl_2$. Heat-stable restriction enzymes were removed using the GeneClean® system and the DNA was dissolved in 20 µl of TA Buffer (Epicentre Technologies). In each case 1 unit of the HK™ Phosphatase was added per µg of vector DNA present. The reaction was incubated at 30 °C for 1-2 hours then a 15 minute 65 °C incubation was performed to inactivate the HK™ Phosphatase. The DNA was removed from the buffer by either ethanol precipitation or GeneClean® and redissolved in $dH_2O$ at appropriate concentration for ligation conditions.

### 2.6.15 Preparation of competent cells for electroporation

The method described in the Bio-Rad Gene Pulser™ instruction manual for the preparation and storage of bacterial cells to be electroporated was followed with

minor modifications. Water used in this procedure was purified by a Barnstead NANOpure apparatus to a resistance of 13.0 MΩ or greater, it was then sterilised by autoclaving.

A single bacterial colony was used to inoculate a 10 ml of LB and was incubated o/n. This inoculum was used to seed 1 L of LB contained in a 2 L conical flask. This culture was grown at 37 °C, 300 rpm, until $A_{600}$=0.8. The cells were chilled on ice then harvested by centrifugation at 4000*g* at 4 °C for 10 min. The cell pellet was washed with 1 L of cold water and centrifuged as before. The cell pellet was then washed with 0.5 L of cold water, re-centrifuged, this step was repeated once more. A final wash was performed with a 20 ml volume of 10% (v/v) glycerol/water and the cells were collected by centrifugation as before. This final cell pellet was re-suspended in 3 ml of ice-cold 10% (v/v) glycerol/water and aliquoted into 40 µl volumes. These competent cells were frozen at -80 °C until required.

### 2.6.16 Electroporation

Competent cells were removed from the -80 °C freezer and defrosted on ice 15 min prior to electroporation. Up to 5 µl of DNA (dissolved in either $dH_2O$ or low ionic strength buffer) was added to the cells before being transferred to a pre-chilled (0 °C) 0.2 cm gap electroporation cuvette.

Electroporation was performed on a Bio-Rad Gene Pulser™ set to 2.5 kV at 25 µF with the Pulse Controller set at 200 Ω. After delivery of a single pulse, the cells were immediately resuspended in 1 ml of SOC medium, transferred to Universal bottles and incubated at 37 °C with gentle shaking (200 rpm) for 1-2 hr. Transformants were plated onto selective media and incubated o/n.

### 2.6.17 Plasmid curing-SDS method

This method was used by Aislabie et al. (1990) to determine which of the plasmids of *P. aeruginosa* QP were associated with quinoline degradation. It is an approach described by Tomoeda et al. (1968).

Approximately $2\times10^8$ QP cells were placed into 100 ml of trypticase soy broth (Difco) amended with 0.06% (w/v) SDS placed in a 250 ml conical flask, these were incubated at 30 °C with gentle shaking for 24 hr. A 1 ml inoculum was transferred to fresh SDS containing medium and incubated as before. After the fifth repetition of this process, aliquots of the cells were plated onto LB agar, 500 colonies were randomly selected and analysed on the quinoline plate assay.

## 2.7 WHOLE GENOME ANALYSIS USING CHEF ELECTROPHORESIS

### 2.7.1 Isolation of genomic DNA

DNA was isolated from *P. aeruginosa* strains immobilised in agarose by an adaptation of the method of Römling and Tümmler (1993).

*P. aeruginosa* was incubated o/n in LB broth to late exponential phase. The bacteria were harvested by centrifugation for 2 min at 12000*g*, then washed twice with SE buffer, approximately $1.5\times10^8$ cells were cast in 60 µl of 1% w/v agarose (Low Melt Preparative grade, Bio-Rad) in a Bio-Rad agarose plug mould. Embedded cells were incubated o/n at 56 °C in lysis buffer. The agarose blocks were transferred to 5 ml of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA pH 8.0) amended with 1mM PMSF, and incubated for 2 hr at room temperature with gentle agitation. This step was repeated with fresh TE/1 mM PMSF. The agarose blocks were washed 3 times with TE buffer, then placed in fresh TE buffer and stored at 4 °C until required.

### 2.7.2 Restriction digestion

Agarose blocks containing genomic DNA were cut into slices of approximately 2 mm in width, each was placed in 150 μl of the appropriate restriction buffer and allowed to equilibrate for a minimum of 30 min at 4 °C. An agarose slice was transferred to fresh restriction enzyme buffer (total volume 150 μl) containing 10 mM dithiothreitol, 20 μg of BSA and 10 U of restriction endonuclease, then incubated at 37 °C o/n. The agarose slice was then equilibrated in 0.5×TBE at 4°C for 30 min prior to electrophoresis.

### 2.7.3 Electrophoresis

A 1% (w/v) agarose gel in 0.5×TBE was cast in the Bio-Rad gel mould (140x128 mm), agarose slices containing endonuclease-treated DNA were placed in the wells and sealed with molten 1% w/v agarose (Low Melt Preparative grade, Bio-Rad) in 0.5×TBE. CHEF electrophoresis was performed on a Bio-Rad CHEF-Mapper™ in 0.5×TBE at 14 °C. The program for electrophoresis was as follows: two state, 28 hr 7 min total run time, included angle 120°, initial switch time of 6.77 sec, final switch time of 44.76 sec (linear ramp) and gradient of 6.0 V/cm.

## 2.8 DNA HYBRIDISATION METHODS

### 2.8.1 DNA probe preparation

Linear DNA was dissolved in 13 μl of $dH_2O$ and melted in a boiling water bath for 10 min then immediately placed in an ice-water bath. This ssDNA was labelled by incorporation of [$\alpha$-$^{32}$P]dCTP with the Boehringer-Mannheim Random Primer Labelling kit according to the manufacturers' instructions. The newly labelled DNA was melted by addition of a 0.1 volume of 1 M NaOH and incubation at room temperature for 5 min. The ss probe could now be added directly to the hybridisation solution.

### 2.8.2 DNA immobilisation onto nylon membranes

DNA was transferred from agarose gels onto nylon membranes using the Pharmacia LKB VacuGene XL apparatus and following the manufacturers' instructions with minor modifications.

Immobilon™-N membranes were pre-wetted with 70% ethanol and rinsed with $dH_2O$ before being placed onto blotting apparatus. The membrane was overlayed with the plastic mask and covered with the agarose gel to be examined. The vacuum pump was adjusted to 50 mbar and the gel covered with the depurination solution for 4 min, the depurination solution was then poured off. The gel was covered with denaturation solution for 3 min, then this solution was discarded. The agarose gel was completely submerged in neutralisation solution for 1 hr while DNA transfer to the nylon membrane was allowed to occur. The vacuum was maintained at 50 mbar throughout the transfer and pretreatment procedure. The nylon membrane was removed from the apparatus and allowed to dry at room temperature, the DNA was then fixed to the membrane using an Ultra-Lum UVC-515 crosslinker delivering 0.12 J/cm$^2$ at 254 nm. Nylon membranes were stored in sealed plastic bags or used immediately in DNA hybridisation experiments.

### 2.8.3 DNA hybridisation

DNA hybridisation, pre-hybridisation and washing steps were all performed in an Hybaid™ Micro-4 hybridisation oven. Nylon membranes, prewetted with 70% ethanol, were washed with $dH_2O$. Membranes were pre-hybridised using pre-hybridisation buffer for 1-3 hours at 68 °C. The pre-hybridisation solution was replaced with hybridisation solution containing the labelled probe. DNA hybridisation was allowed to occur at 68 °C o/n. Denhardt's reagent or BLOTTO were included in the hybridisation protocol appropriately, ie: Denhardt's reagent was added when low-abundance target sequences were involved such as probing chromosomal sequences, BLOTTO was used when plasmid clones were targets (as suggested in Sambrook et al. (1989)).

### 2.8.4 Removal of unbound probe

The membranes were washed in the hybridisation tubes with 100 ml of 2×SSC, 0.1% (w/v) SDS at room temperature for 15 min. The membranes were then washed twice (15 min each) with 100 ml volumes of 0.2×SSC, 0.1 % (w/v) SDS at 68 °C. Membranes were then wrapped in plastic wrap before being examined by autoradiography or the Storm™ 840 system.

## 2.9 COLONY HYBRIDISATION

The protocol described in Sambrook et al. (1989), with modifications, was used for screening bacterial colonies by hybridisation. Hybridisation and membrane wash steps were performed in a Hybaid™ Micro-4 hybridisation oven.

### 2.9.1 Bacterial colony transfer and lysis

LB agar plates (amended with appropriate selection) were patch-inoculated with 100 colonies per 90 mm plate using sterile toothpicks and in duplicate. The bacterial colonies were allowed to grow at 37 °C for 12 hours. Pre-wetted (as described in 2.8.3 above) Immobilon™-N membranes (cut to fit the internal diameter of the agar plates) were placed on a replica plating device. One of the duplicate agar plates, containing the bacterial colonies to be tested, was placed on the membrane and pressed firmly. The membranes were gently peeled off the agar plates and transferred sequentially to a series of trays containing Whatman® 3MM paper saturated with various solutions as follows: 10 % (w/v) SDS, 3 min; denaturation solution, 5 min; neutralising solution, 5 min; and 2× SSC for 5 min. Between each transfer the underside of the membranes were scraped gently against the edges of the trays to remove the excess reagents. The filters were allowed to dry for 1 hr at room temperature before being baked at 80 °C under vacuum for 1 hour on a Hoefer gel dryer (Drygel Sr. SE1160). Membranes were stored sealed in plastic bags until required.

### 2.9.2 Hybridisation

The nylon membranes were pre-wetted (as described in 2.8.3), then placed in 2× SSC solution for 5 min. Membranes were then incubated in 200 ml of pre-wash buffer and gently agitated at 50 °C for 30 min. Following this incubation, the remaining debris from the bacterial colonies was gently wiped away with nappy liners soaked in pre-wash buffer. The membranes were transferred to Hybaid™ tubes (5 per tube) separated by nylon mesh to prevent overlap of the membranes, and pre-hybridised with 100 ml of hybridisation solution in the Hybaid™ oven at 68 °C. The pre-hybridisation solution was discarded and replaced with 15 ml of fresh hybridisation solution containing the labelled probe, membranes and labelled probe were allowed to hybridise o/n at 68 °C.

### 2.9.3 Removal of unbound probe

The membranes were washed in 100 ml volumes of 2× SSC, 0.1% (w/v) SDS at room temperature, this wash step was repeated 3 further times. The final wash was performed in 1× SSC, 0.1% (w/v) SDS at 68 °C for 90 min. For all washing steps the membranes were placed in hybridisation bottles in the Hybaid™ oven.

Membranes were placed on Whatman® 3 MM, wrapped in clingfilm, then analysed by autoradiography or the Storm™ 840 system.

## 2.10 DNA SEQUENCE ANALYSIS OF DOUBLE STRANDED DNA TEMPLATES

DNA sequence analysis of double stranded templates was performed either manually, using the dideoxy chain termination method (Sanger et al. 1977), or using an ABI Prism Model 377 version 3.0 automated sequence apparatus employing dRhodamine terminator chemistry, at the Waikato DNA Sequence Facility (Department of Biological Sciences, the University of Waikato). Manual sequence reactions were performed using the T7 DNA polymerase

Sequenase® Version 2.0 kit (United States Biochemical) and labelling the DNA with [α-$^{35}$S]dATP (Amersham, SJ1304).

### 2.10.1 DNA template preparation

The DNA sequence to be analysed was ligated into pUC19 and replicated in *E. coli* DH5α. Typically single colony inoculations of the appropriate colony were made into 10 ml volumes of LB /Ap and grown o/n. Plasmid DNA was isolated by the lysis by boiling method (2.6.5). The DNA was purified using the GeneClean® system. An aliquot of the DNA was digested with an appropriate restriction enzyme, to ensure quality of the sample, and analysed by AGE. Purity and concentration of the DNA sample were then determined. DNA was then used in manual or automated sequencing experiments.

## 2.11 SEQUENASE® DNA SEQUENCE REACTIONS

DNA sequence reactions were performed with a Sequenase® Version 2.0 kit from United States Biochemical. DNA was prepared from *E. coli* DH5α

### 2.11.1 Annealing of primer to template

A 3-5 μg aliquot of the DNA template was brought to a volume of 32 μl with $dH_2O$. The double stranded DNA template was denatured by the addition of 8 μl of 2M NaOH followed by a 10 minute incubation at room temperature. This ssDNA was precipitated by addition of 7 μl of 3 M sodium acetate, 4 μl of $dH_2O$ and 120 μl of ice cold ethanol then incubated at -80°C for 15 min. The DNA was collected by centrifugation in a microfuge tube, washed with 70% ethanol then briefly air dried. The DNA was dissolved in 7 μl of $dH_2O$, 1μl of the primer DNA (30 pmol for 17 base primer) and 2 μl of the Sequenase® reaction buffer were then added. The solution was mixed well and incubated by placement of the microfuge tube into a 100 ml volume of water at 65 °C for 2 min, the tube was then allowed to reach room temperature over a period of 30 min by

allowing the beaker to cool. The annealed DNA was then placed on ice before being labelled.

### 2.11.2 Labelling reactions

The Labelling Mix was diluted 5 fold with $dH_2O$, 2.0 µl of this was added to 10.0 µl of the template/primer solution, 1.0 µl of 0.1 M DTT, 0.5 µl of [$\alpha$-$^{35}$S]dATP and 2.0 µl of Sequenase® Version 2.0 T7 DNA polymerase (first diluted 1:8 in ice cold enzyme dilution buffer) were then added and mixed on ice. This reaction mix was incubated at room temperature for 2-5 min to allow incorporation of the [$\alpha$-$^{35}$S]dATP into the nascent DNA produced by the polymerase extension of the template annealed primer.

### 2.11.3 Termination reactions

The four dideoxynucleotide mixes (2.5 µl of each) were transferred to separate 0.5 ml-microfuge tubes previously warmed to 37 °C and labelled "G", "A", "T" and "C". To each of these, 3.5 µl of the completed labelling reaction mix was added with mixing, the solutions were allowed to react at 37 °C for 3-5 mins to allow termination of nascent strand synthesis by incorporation of the dideoxynucleotides. Following this incubation, the termination reaction was stopped by addition of 4 µl of the stop solution to each of the 4 tubes, the contents of the tubes were mixed thoroughly and collected by a brief centrifugation. The resulting solutions were then analysed by electrophoresis or stored at -20 °C.

### 2.11.4 Sequencing gel electrophoresis

Sequencing acrylamide gel electrophoresis was performed on a Bio-Rad Sequi-Gen® II Nucleic acid Sequencing cell (21 x 40 cm). The glass plates and mould were carefully cleaned and the bonded glass plate was treated with Sigmacote® to prevent sticking of the gel to this plate. A 5% (w/v) acrylamide gel (Appendix II) was cast in the apparatus using 0.4 mm spacers. The gel was pre-run for approximately 60 minutes at 1500-1800v such that the gel temperature reached

50 °C. Samples were denatured at 95 °C before being loaded into a sharkstooth comb. Electrophoresis was performed at a voltage to maintain a gel temperature of 50 °C until the blue dye reached the bottom of the gel. A second set of the same samples could then be loaded and electrophoresed until the blue dye again reached the bottom of the gel, this would achieve greater separation and therefore generate more sequence data. Following electrophoresis, the front glass plate was removed from the apparatus with the gel still attached and transferred to a bath of 5% (v/v) acetic acid 15% (v/v) methanol/d$H_2O$. Gentle agitation of the plate for 15 min ensured removal of urea from the gel. The gel was removed from the glass plate and transferred to Whatman 3MM paper, dried on a Hoefer gel dryer (Drygel Sr. SE1160) at 80 °C, under vacuum for up to 2 hr and then examined by autoradiography or the Storm™ 840 system.

### 2.11.5 Sequence similarity searches

Sequence similarity comparisons between sequences derived from QP100 and QP100-8 and those stored at the NCBI (National Centre for Biotechnology Information) were performed using the blastn and blastx algorithms (Altschul et al. (1990) & Altschul et al. (1997)) (at web site: www.ncbi.nlm.nih.gov). Searches were also performed using the FASTA algorithm (Pearson and Lipman 1988) (at website: www.embl-heidelberg.de).

### 2.11.6 Sequence data analysis

DNA sequence data including nucleotide composition, putative open reading frame searches and restriction analysis was examined using DNAMAN version 2.5 Lynnon BioSoft.

## 2.12 AUTORADIOGRAPHY

Membranes labelled with [$\alpha$-$^{32}$P]dCTP and wrapped in cling-wrap were exposed to Amersham Hyperfilm™ MP at -80 °C in X-ray cassettes. Acrylamide DNA sequence gels labelled with [$\alpha$-$^{35}$S]dATP were exposed directly to Amersham

Hyperfilm™ MP or Amersham Hyperfilm™ $^{35}S$ sequencing film at room temperature in X-ray cassettes.

Exposed films were developed in Agfa G150 X-ray film developer for 5 min, rinsed briefly in water and fixed in Agfa X-ray film fixer for 5 min. Following processing, the autoradiographs were rinsed in running water and allowed to air dry.

## 2.13 THE STORM™ SYSTEM FOR PHOSPHOR-IMAGING OF RADIOACTIVE MEMBRANES

The Molecular Dynamics Storm™ 840 instrument was used to record images from Kodak Storage Phosphor screens which had previously been exposed to [$\alpha$-$^{32}P$]dCTP labelled membranes or [$\alpha$-$^{35}S$]dATP labelled sequencing gels. The resulting data was processed using the Molecular Dynamics ImageQuant™ software package.

Membranes were wrapped in plastic wrap and exposed to Phosphor screens at room temperature for between 30 min and o/n. Typically a membrane displaying >1000 cpm, on a hand-held monitor, would require an exposure time of 30 min for an image to be recorded. Sequencing gels were exposed to Phosphor screens for 24 hours.

## 2.14 ISOLATION AND IDENTIFICATION OF COMPOUNDS EXTRACTED FROM QBHS CULTURE MEDIUM

### 2.14.1 Extraction of ethyl acetate soluble material

Single colony inoculations of QP100 mutants were made into 100 ml volumes of QBHS medium amended with 0.2% w/v glucose. The cultures were incubated at 30 °C (200) rpm for 24-36 hours. The UV spectrum of the growth medium was examined periodically during this incubation. Once the UV

spectrum matched that expected of the mutant, precipitated matter and the bacterial cells were removed from the medium by centrifugation at 12000*g* for 2 min. The supernatant was brought to pH 7 then extracted with an equal volume of ethyl acetate in a separating funnel. Two further ethyl acetate extractions were performed and the ethyl acetate fractions were pooled. The ethyl acetate was removed by a rotary evaporator and the resulting crystalline product was dissolved in 15% (v/v) methanol/d$H_2O$.

### 2.14.2 HPLC analysis of extracted compound

Samples of the extracted compound were analysed on a Shimadzu LC-10AT Liquid Chromatogram. The sample was eluted through a reversed phase C18 column using a water/methanol isocratic mobile phase of varying composition. Eluted products were assayed using a Shimadzu SPD-M10A diode array detector.

### 2.14.3 NMR analysis of extracted compound

Proton and carbon NMR analyses were performed on a Varian Unity instrument operating at 300 MHz and 75 MHz respectively, at the Department of Chemistry, University of Canterbury.

# 3. RESULTS

## 3.1 EFFECT OF MEDIA COMPOSITION ON QUINOLINE DEGRADATION BY QP

The aim of this experiment was to find the medium which supported the fastest degradation of quinoline by QP cells.

Medium C allowed QP to degrade quinoline the fastest, no quinoline remained in this medium after 20 hours incubation (Figure 3-1). Quinoline degradation was consistently marginally slower results in Medium B. QP cells incubated in Medium A for 20 hours produced a pink pigment with a UV spectrum identical to that of 2-hydroxyquinoline. Complete quinoline degradation required incubation for a further 12 hours. Medium C (Bushnell-Haas & trace elements with 0.03% (v/v) quinoline as the sole source of carbon and nitrogen (QBHS)) was selected as the medium for incubating QP strains in the presence of quinoline.

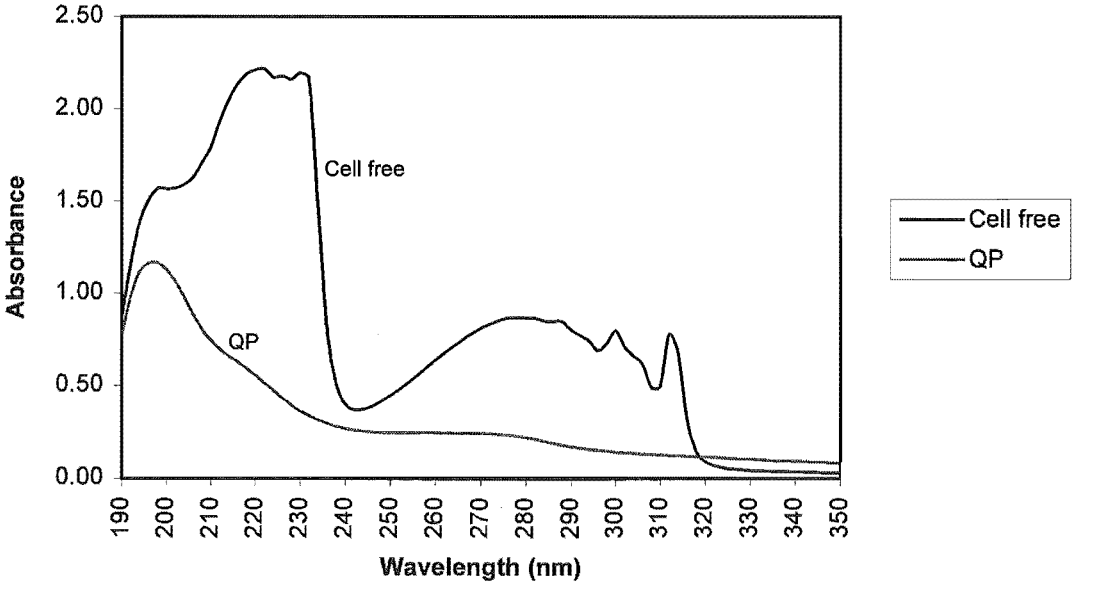


**Figure 3-1. Overlayed UV spectra of QBHS medium following a 20 hour incubation at 30°C with QP cells and a cell free control.**

The results from Figure 3-1 are consistent with QP cells degrading quinoline to a concentration below the background UV absorbance of the medium as the UV spectrum of the cell free control is that expected of quinoline. As expected, the turbidity of the medium in the flask increased over the incubation period as the quinoline disappeared.

Molybdenum salts are required by bacteria degrading quinoline (Bauder et al. 1990; Blaschke et al. 1991). This element was shown to be an integral component of the cofactor of quinoline oxidoreductase in a *Rhodococcus* sp. and *P. putida*. If QP strains utilised a similar enzyme system, then molybdenum would also be required for growth on quinoline. Since tungstate is an antagonist of molybdenum (Blaschke et al. 1991), it was added to the quinoline culture medium of QP. Tungstate did inhibit QP growth on quinoline (Figure 3-2) in a manner consistent with that observed by Bauder et al. (1990) and Blaschke et al. (1991).

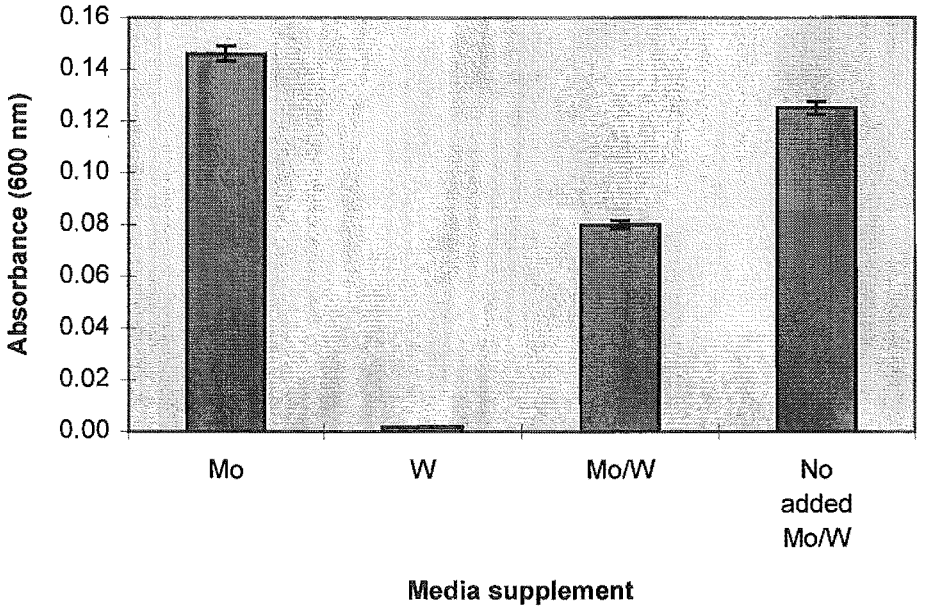


**Figure 3-2 Relative turbidity ($A_{600}$) of 4 cultures of QP cells grown in QBHS with various supplementations of molybdate and tungstate.** Molybdate and tungstate added at $10^{-7}$ M. Error bars indicate 1 standard deviation.

## 3.2 DEVELOPMENT OF A PLATE ASSAY TO SCORE QUINOLINE DEGRADING DEFICIENT MUTANTS

A stringent plate assay would allow for the rapid screening of large numbers of colonies, following mutagenesis, to evaluate their ability to grow on quinoline as a sole source of carbon, nitrogen and energy. As this assay would indicate inability to degrade quinoline by failure of the colony to show visible growth, it was necessary that the growth rate of the colonies be consistent over the entire surface of the plate to minimise false negatives, such as poor growth of non-quinoline mutants.

Quinoline did not dissolve readily in media formulations, the first task was, therefore, to determine how quinoline could be most consistently provided to cells. Table 3-1 is a summary of these methods.

**Table 3-1 Quinoline plate assay.** Comparison of various quinoline application methods.

| Method | Observation | Relative growth rate of colonies |
|---|---|---|
| **(1) Application in ether** quinoline deposited on the plate's surface in a volatile solvent | Colonies grew with uneven edges, often growth of colonies seemed inconsistent over plate surface. Colony maximum size appeared smaller than in (3) below. | ++ |
| **2) Vapour method** quinoline provided as a vapour in the headspace of the plate | Colonies directly over the quinoline spot grew more rapidly and larger than those toward the edge of the plate. | + to +++ |
| **3) Emulsification method** quinoline solubilised in Tween 80/d$H_2O$ | Even colony growth over entire plate surface, colonies grew to maximum size (2-3mm with 30 inoculations/plate) within 48 hr. | ++++ |

While all the 3 methods proved to supply quinoline in a utilisable way to QP cells, method 3 was adopted because the growth rates was highest and homogenous over the entire surface of the agar plate. QP cells were also

tested for their ability to utilise Tween 80 as a carbon source and were found unable to do so. Other *P. aeruginosa* lab strains, which served as negative controls for these assays, failed to grow on these plates, as expected.

Quinoline was added to plates at a concentration of 0.03% v/v (approximately 2.5 mM) this is the maximum quinoline concentration without growth inhibition for QP (Aislabie et al. 1990). This concentration supported the growth of about 30 colonies per plate. If the number of colonies per plate was increased to 50 colonies, the growth-rate of the colonies was retarded and colonies were smaller (presumably due to limiting quinoline concentration) making rapid interpretation of results more difficult.

### 3.2.1 The quinoline plate assay

The quinoline plate assay was performed in QBHS medium solidified with 1.5% (w/v) agar with quinoline solubilised in Tween 80. Typically 28 colonies were tested per plate for their ability to grow on quinoline. Both a negative prototrophic *P. aeruginosa* and positive control (QP100) per plate. The plates were sealed with cling-wrap and incubated at 30 °C.

A typical plate assay result appears in Figure 3-3. The site of $Qln^-$ inoculation can be seen toward the centre of the plate. No growth is visible around the point of inoculation as with the negative control colony (bottom left). The growth of the positive control inoculation is indistinguishable to those under test.

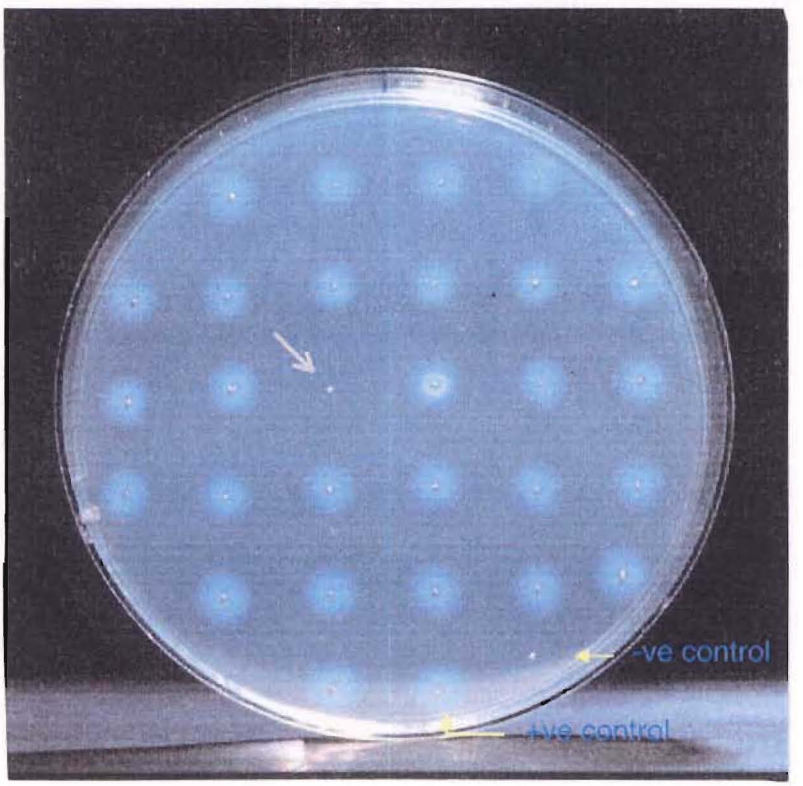


**Figure 3-3. Plate assay.** Both positive (QP100) and negative controls are labelled, putative $Qln^-$ inoculation is marked with a grey arrow.

## 3.3 PLASMID ANALYSIS

Aislabie et al. (1990) had shown that QP harboured 4 large plasmids (180 to 320 kb). The 320, 225 and 250 kb plasmids were shown by plasmid curing experiments to be associated with quinoline utilisation by this organism. The presence of plasmids, however, could not be detected in a later study by Horridge (1991) using the identical plasmid preparation techniques described in Aislabie et al. (1990).

### 3.3.1 Plasmid isolation

The presence of plasmids in QP has remained an unresolved issue. It was considered important to determine whether the quinoline degradation phenotype was conferred by plasmids in QP as, for example, specific

techniques to isolate genes on plasmids can be performed. The results in (Aislabie et al. 1990) suggest at least some of the genes involved with quinoline metabolism exist in duplicate on at least two plasmids. This would make transposon mutagenesis a poor choice for isolating mutants of these genes. In a further attempt to determine whether QP contained plasmids, the plasmid isolation technique described by Kieser (1985) was performed and plasmid curing experiments described in Aislabie et al. (1990) were repeated on QP. The plasmid preparation of Kieser (1985) was chosen because this method has been shown to be successful in the isolation of large plasmids from various environmental isolates of *Pseudomonas* species capable of hydrocarbon, PAH and heterocyclic compound degradation (Foght and Westlake 1988).

Plasmid DNA was extracted from cultures of QP, both *P. aeruginosa* OT899 (RP4), and *A. tumefaciens* C58 served as control strains, as described in methods chapter.

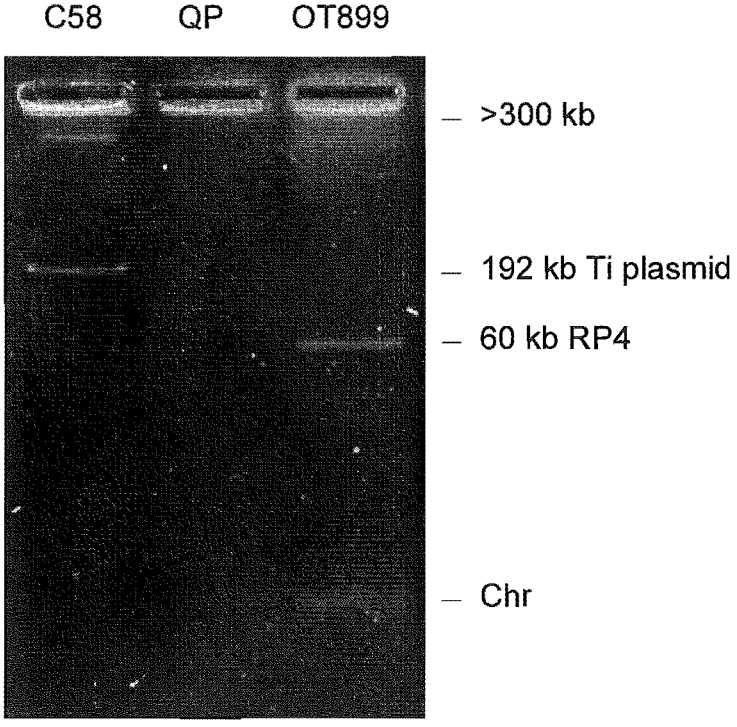


**Figure 3-4. Plasmid analysis of *A. tumefaciens* C58, *P. aeruginosa* QP and *P. aeruginosa* OT899.** Chr is fragmented chromosomal DNA. **Electrophoresis was performed on the large format apparatus, 50V at 4 °C for 17 hr.**

It can be observed from Figure 3-4 that the 192 kb Ti plasmid and the 300 kb cryptic plasmid from C58 are clearly visible. Similarly, in OT899, the 60 kb RP4 plasmid is also visualised by this method. No detectable plasmid DNA from QP has been isolated using this technique, the results of this gel are typical for many plasmid isolation attempts using this method. The result in Figure 3-5 show this method is successful in the isolation of RP4 in the QP101 background, again no evidence of other plasmids harboured by QP101 are visible in this gel.

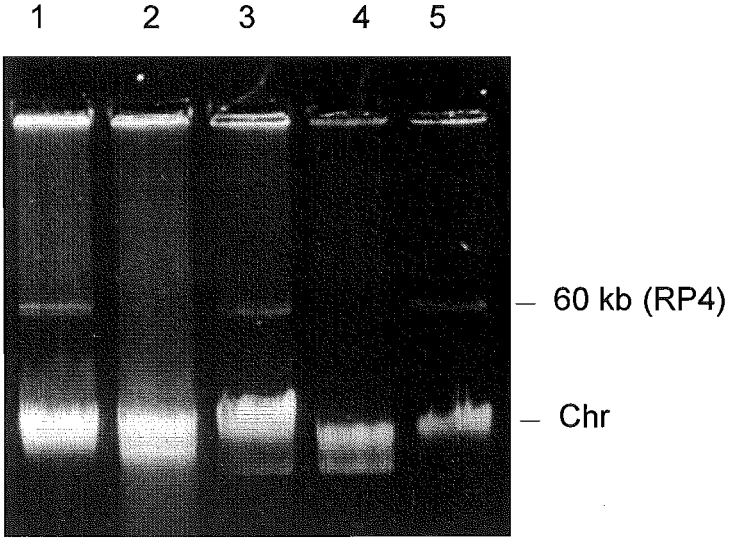


**Figure 3-5. RP4 isolation in various *P. aeruginosa*.** Lanes are as follows: 1, OT684 (RP4); 2, OT684; 3, QP101 (RP4); 4, QP101; 5, OT899 (RP4).

### 3.3.2 Plasmid curing

The SDS method of plasmid curing described in Aislabie et al. (1990) was repeated with QP. Following 5 serial transfers of the QP culture through SDS-containing medium, a dilution of the resulting cells was plated onto LB agar plates. Five hundred colonies were patched onto the quinoline assay plates. All of these colonies grew on the assay plates and therefore had not lost the quinoline degrading phenotype. These results contrast with those observed by

Aislabie et al. (1990), where 200 colonies were screened in this manner and 6 cured derivatives were isolated displaying a spectrum of plasmid loss and various in their abilities to degrade quinoline. It was concluded from this result that the quinoline degrading phenotype in QP was now stable in the presence of SDS.

### 3.3.3 Whole genome CHEF electrophoresis analysis of QP

A whole genome analysis of QP was performed using the Bio-Rad CHEF-Mapper™ electrophoresis apparatus. These experiments were performed with the aim of comparing the *Spe*I fragmentation pattern of QP to that of other *P. aeruginosa*. This methodology would also reveal the presence of large plasmids. Genomic DNA was liberated from QP and OT903 and digested with *Spe*I (Figure 3-6) as described in methods 2.7.

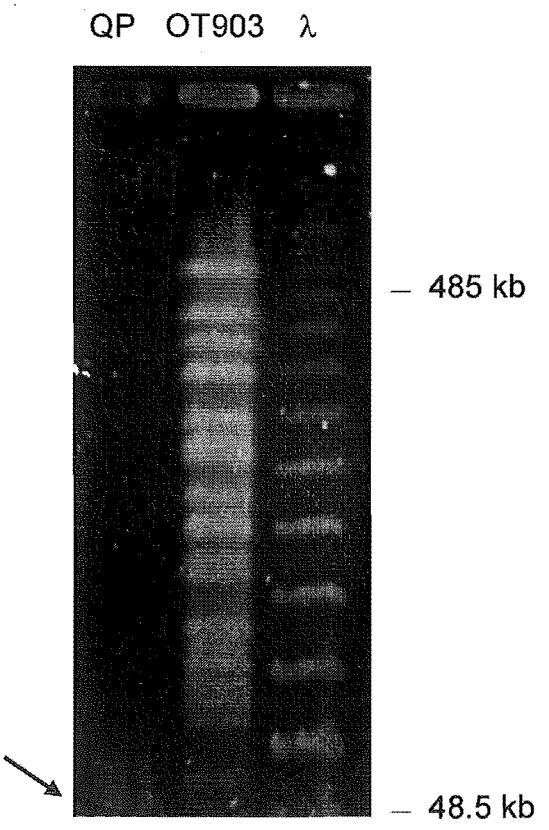


**Figure 3-6. *Spe*I digest of QP and OT903 genomic DNA.** Arrow indicates fragmented low molecular weight QP DNA. λ= concatemer of λ genome molecules (Bio-Rad).

While OT903, a wild-type clinical isolate, gives a *Spe*I banding pattern expected of *P. aeruginosa*, QP DNA appears to have been totally digested. Several attempts at this protocol failed to extract large fragments of QP DNA, Figure 3-6 gives a typical result were DNA is fragmented to smaller than 50 kb.

To investigate whether this apparent fragmentation of QP was exonuclease mediated, and therefore might be prevented by heat treatment, the following experiment was performed. QP and OT2 (a control) cells suspended in agarose were incubated at 75 °C for 10 min before DNA extraction as described in methods (2.7). Half of the liberated DNA samples were treated with *Spe*I, the remainder were not treated with restriction enzymes.

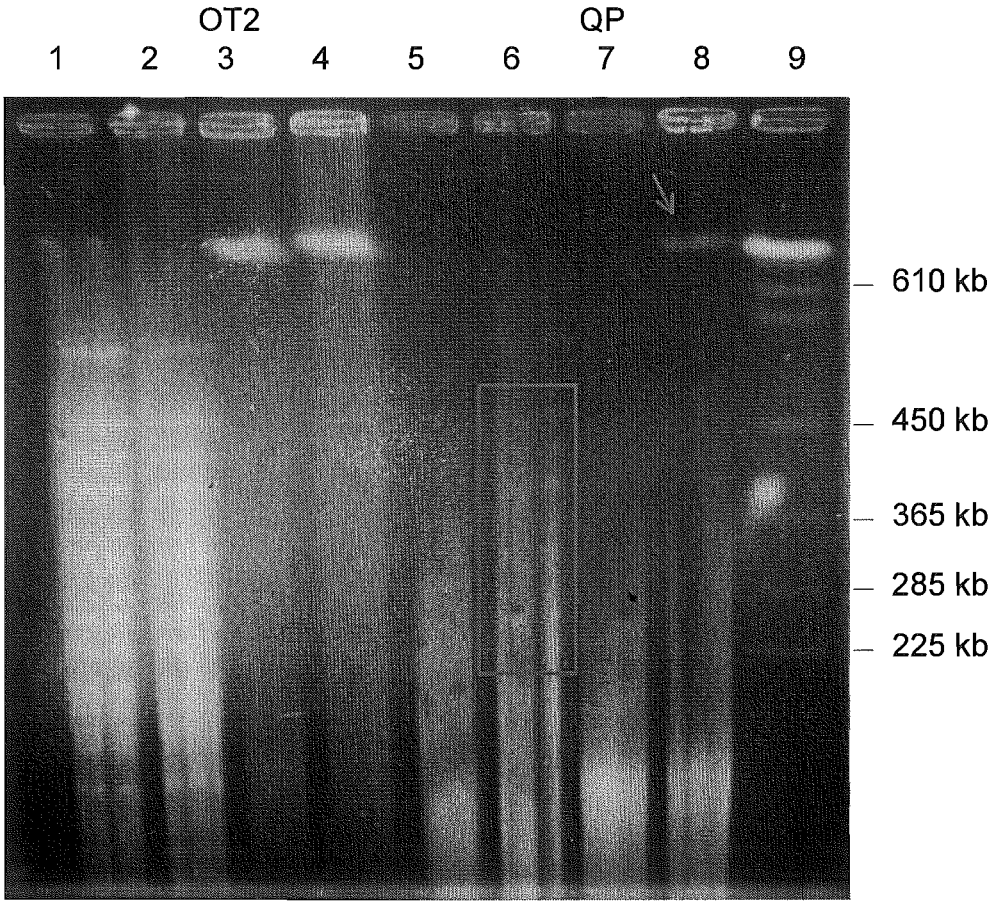


**Figure 3-7. Genomic preparations of *P. aeruginosa* OT2 and QP with and without heat treatment.** Key: 1, *Spe*I OT2; 2, *Spe*I OT2 (heat treated); 3, OT2 undigested; 4, OT2 undigested (heat treated); 5, *Spe*I QP; 6, *Spe*I QP (heat treated); 7, QP undigested; 8, QP undigested (heat treated); 9, Yeast chromosome size marker.

Heat treatment of OT2 cells prior to lysis had little, if any, effect on either the unfragmented genome or the *Spe*I digested DNA (Figure 3-7). QP cells lysed without heat treatment provided an expected pattern of DNA digestion with no intact genome band, as seen in the undigested OT2 lane. Heat treatment of non-restriction enzyme treated QP cells preserved some of the intact genomic DNA (grey arrow) at the same size as OT2 genomic DNA. The *Spe*I digest of this DNA shows some evidence of the larger *Spe*I fragments surviving (inside the grey box), these bands appear to correspond to those observed in OT2. This result provides evidence for the potential of this technique to have application in QP strains.

## 3.4 TRANSPOSON MUTAGENESIS- METHOD DEVELOPMENT

### 3.4.1 Initial Tn*5* mutagenesis experiments

Initial experiments with Tn*5* (and Tn*5*-B21) and QP strains resulted in failure to isolate any mutants of QP100 or QP101 (using the method described in 2.5.2). Various mating experiments were attempted with *E. coli* donor strains but failed to provide transfer of transposon antibiotic resistance determinants to QP100. If mating periods were extended to 20 hours, QP100 cells grew thickly on the filters and displayed resistance to antibiotic markers. These colonies, however, were of pleiomorphic colony morphology and displayed heterogeneous growth rate. Several thousand of these colonies were screened from various mating experiments for auxotrophy. No auxotrophic mutants were isolated in these experiments indicating failure to mutagenise QP100. Control experiments where QP100 cells were incubated without the donor cells, also provided similar antibiotic resistant cells. Both of these results suggested that the antibiotic resistant colonies had arisen spontaneously and not as a result of transposon insertion.

### 3.4.2 RP4 mating experiments

To determine whether failure to infect QP100 with the transposon was a result of failure in delivery of the transposon, various RP4 mating experiments were performed. The transposon is delivered via a suicide plasmid (pSUP derivative) which is incapable of replication in *P. aeruginosa* and is not self transmissible, therefore, expression of the antibiotic marker from the transposon is evidence that the transposon has incorporated into the genome of its new host. The pSUP plasmids are transferred from their host *E. coli* S17 (or SM10) using RP4 transfer functions provided in *trans* from an RP4 molecule incorporated in the host's chromosome. The pSUP family of plasmids contain the Mob site from RP4, the plasmid is transferred from its host to the cell to be mutagenised using the RP4 conjugation machinery (Simon et al. 1989) and (Simon et al. 1983). A successful transfer of RP4 into QP100 would indicate that these conjugation functions are functional with QP100 and, therefore, the pSUP plasmid system has application for delivery of the transposon in this organism.

The following crosses (Table 3-2) were performed in liquid medium as described in methods (2.5.1) section.

**Table 3-2. Frequency of intra and interspecific RP4 plasmid transmission.**

| RP4 donor (strain) | recipient (strain) | frequency[a] |
|---|---|---|
| *E. coli* (AB1157) | *P. aeruginosa* (QP100) | ND |
| *P. aeruginosa* (OT899) | *P. aeruginosa* (QP100) | $4\times10^{-3}$ $\pm2\times10^{-4}$ |
| *P. aeruginosa* (QP101) | *P. aeruginosa* (QP100) | 1.6 $\pm7\times10^{-2}$ |

[a]All frequencies are expressed as transconjugants per limiting parent (donor).
ND; none detected

No transmission of RP4 from *E. coli* to QP100 could be detected in these experiments (ie. $\leq2\times10^{-7}$ transconjugants per limiting parent). However, RP4 can be transmitted to QP100 by another donor, *P. aeruginosa* (OT899), at least $2\times10^{4}$ times more efficiently than by *E. coli* and with QP101 as a donor, a further 400 times more efficiently than OT899. It would seem a restriction

barrier exists between *E. coli* and QP100 and to a lesser extent between OT899 and QP100. It can be concluded though, if this apparent restriction barrier is overcome, that RP4 can be successfully transmitted into QP100.

### 3.4.3 Tn*5* B21 transposon mutagenesis of QP100

The transposon Tn*5* was chosen for the mutagenic agent in these experiments due to its relatively high rate of transposition, low target sequence specificity and broad host range (Berg 1989). As with other *P. aeruginosa*, QP strains have a high innate tolerance to kanamycin (unpublished observation). Fortunately several engineered Tn*5* transposons have been produced which confer resistance to many other antibiotics (Simon et al. 1989). The Tn*5* derivative Tn*5*-B21 was selected as the mutagenic agent. This engineered transposon confers tetracycline resistance to its host (Figure 3-8).

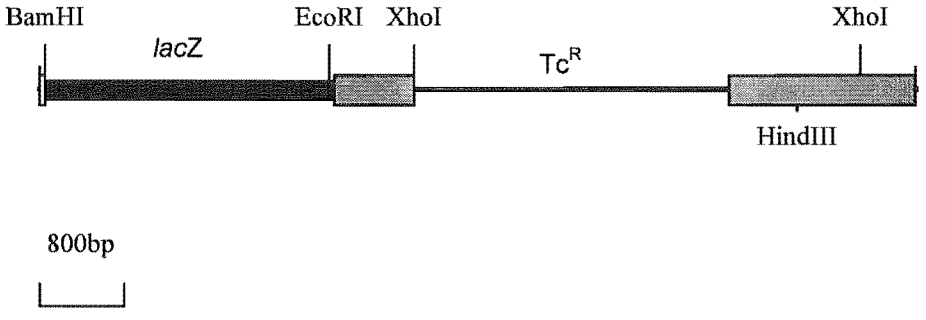


**Figure 3-8. Physical map of Tn*5*-B21 showing selected restriction sites.** From Simon et al. (1989). Light grey boxes indicate Tn*5* DNA.

The transposon was delivered by a suicide vector (pSUP102) introduced into QP strains by conjugation (section 3.4.2). The results of the RP4 mating experiments, along with the transformation experiments with pRO1614 (section 3.9.2), had indicated that a restriction barrier exists between *P. aeruginosa* QP strains and *E. coli* and to a lesser extent with other *P. aeruginosa* PAO derived strains. Experiments by Holloway (1965) had shown that the restriction barrier in *P. aeruginosa* could be partially overcome by growing the organism at 43 °C

for at least 5 generations. This phenomenon has been applied successfully (Rombel and Lamont 1992) to increase the transmission frequencies in *P. aeruginosa* and *E. coli* S17 (pSUP202) crosses. QP100 was treated in a similar manner in the following mutagenesis experiments.

Transposon (Tn5-B21) mutagenesis was performed on QP100 cells as described in methods (2.5.3). Approximately 600 $Rf^R/Tc^R$ colonies were recovered per mating experiment of which a total of 1100 colonies were recovered from two separate experiments.

### 3.4.4 Quinoline-plate assay analysis of the QP100 mutants

The $Rf^R/Tc^R$ transconjugant colonies from matings of QP100 with *E. coli* S17 (pSUP102-Gm Cm::Tn5-B21) were inoculated sequentially to quinoline-assay plates then a fresh LB/Tc rescue plates using a sterile toothpick. The LB/Tc plates allowed for the recovery of putative quinoline degrading deficient mutant ($Qln^-$) colonies were the equivalent colonies did not grow on the quinoline plate assay.

The quinoline-assay plates were incubated for 48 hours during which allowed the control QP100 colonies to grow approximately 2-3 mm beyond site of inoculation. During this period, 9 $Rf^R/Tc^R$ transconjugant colonies did not grow on these assay plates. These mutants were designated QP100-1 to QP100-9 inclusive. To determine whether these mutants were auxotrophic but not $Qln^-$, the corresponding colonies from the LB/Tc plate were transferred to M9-agar plates supplemeted with glucose. After 24 hours incubation at 37 °C, QP100-2, QP100-3, QP100-4 and QP100-5 did not grow on the M9 agar plates, these were likely auxotrophs of no particular interest here. indicating auxotrophy. The putative $Qln^-$ mutants (QP100-1, QP100-6, QP100-7, QP100-8 and QP100-9), which grew on the M9-agar plates, were subject to verification in liquid medium with spectrophotometry.

### 3.4.5 Liquid media spectral assay of the putative Qln⁻ mutants

To determine whether the putative Qln⁻ mutants were no longer able to metabolise quinoline to any extent, and were not just growing slowly (ie 'less fit", but unaffected in their ability to degrade quinoline), the mutant cells were inoculated into QBHS medium amended with quinoline. This also served as a double-check for the putative Qln⁻ mutants, as weak growth on the assay plate may have arisen because of poor inoculum. The disappearance/modification of quinoline by these cells could then be monitored spectrophotometrically (Figure 3-9).

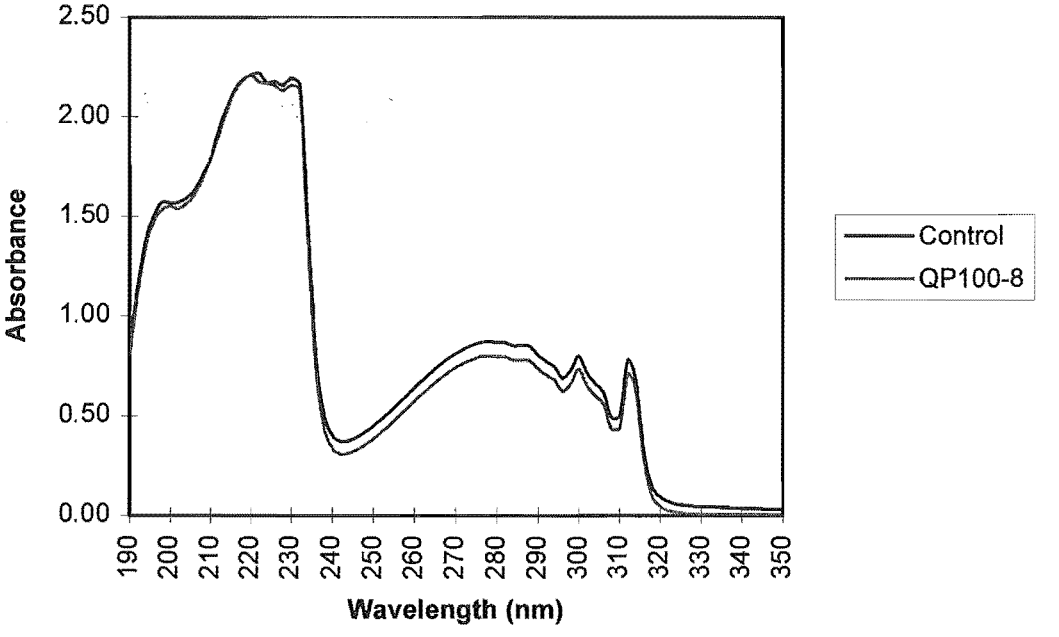


**Figure 3-9. Overlayed UV spectra of QBHS following incubation (1 week) with QP100-8 cells and the uninoculated control.** This spectrum is representative of what was observed with both QP100-6 and QP100-7.

The spectra in Figure 3-9 shows that QP100-8 (and QP100-6 and QP100-7) was no longer able to degrade quinoline (Qln⁻). The Qln⁻ phenotype is stable in these mutants as this spectrum remained unchanged throughout the 2 week

period this experiment was conducted. The flasks never became turbid throughout the experiment indicating failure of the QP100 mutants to grow.

By contrast, the mutants QP100-1 and QP100-9 both retained the ability to degrade quinoline and both degraded quinoline slower that the QP100 parent. QP100-9 was incubated for 30 hr before the UV spectrum of the growth medium resembled the observed spectrum of QP100 at only 20 hr under the same conditions (Figure 3-1). QP100-1 was slower than QP100-9, taking approximately 60 hr to arrive at the same observed spectrum. Turbidity increased in these cultures over the incubation period. It was concluded that QP100-1 and QP100-9 could still grow on quinoline and where not studied further.

## 3.5 CHARACTERISATION OF THE MUTANT PHENOTYPE OF QP100-6, QP100-7 AND QP100-8

### 3.5.1 UV spectra of glucose supplemented QBHS medium inoculated with QP100 mutants

The mutants QP100-6, QP100-7 and QP100-8 were identified as being quinoline degrading deficient ($Qln^-$) as they failed to degrade or grow on quinoline. Since QP strains can metabolise quinoline in the presence of another carbon source, the mutants' ability to grow in QBHS medium supplemented with both glucose and quinoline could be tested. The addition of glucose would allow the cells to grow only if the transposon had inserted in a sequence of DNA that was necessary for quinoline degradation further in the pathway, post-nitrogen removal from the heterocyclic ring. Failure of the cells to grow in this medium without supplementary nitrogen would indicate the transposon insertion had affected a step in the metabolism of quinoline prior to nitrogen removal from the heterocyclic ring.

Each of 3 flasks containing 100 ml of QBHS/glucose was inoculated with a single colony of each of the 3 mutants and incubated for 1 week at 30 °C (with aeration). Samples of the culture were taken daily and their UV spectra analysed (Figure 3-10).

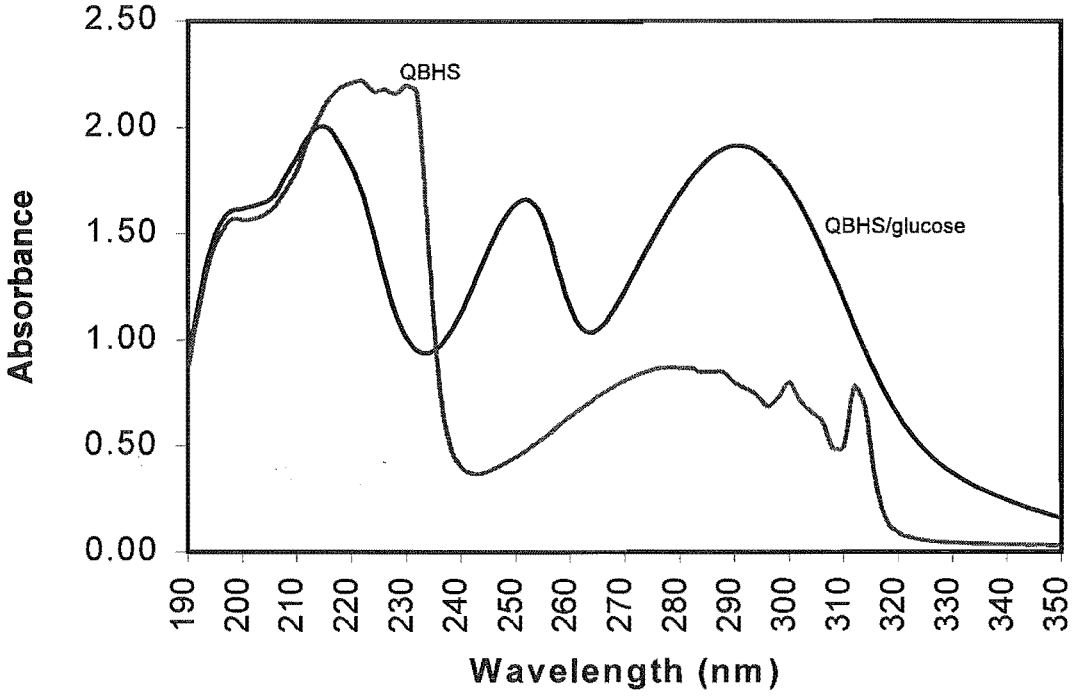


**Figure 3-10. Overlayed spectra of QBHS medium and QBHS/glucose both following incubation with QP100-8 for 48 hr.** This spectrum is representative of what was also observed with both QP100-6 and QP100-7.

It appears from Figure 3-10 that all three mutants accumulated the same compound(s) in their growth medium. These spectra remained unchanged for the duration of the experiment (1 week). The presence of glucose in QBHS allowed the QP100 mutants to grow as turbidity increased in the culture medium over the incubation period.

### 3.5.2 Isolation and identification of the accumulated compound

As indicated by the stable UV spectrum ($\lambda_{peak}$=212, 252, 292nm) each mutant accumulated the same intermediate(s). Analysis of this intermediate compound

was restricted to QP100-8. A single colony of QP100-8 was inoculated into QBHS medium supplemented with glucose and was incubated until the examined UV spectrum matched the observed spectrum in Figure 3-10 (approximately 48 hr). After centrifugation to remove cells and solid mater, organic soluble compounds were extracted using ethyl acetate. The ethyl acetate was removed by rotary evaporation and the remaining white crystalline solid was dissolved in methanol/water (15% v/v). An aliquot of this solution was diluted with $dH_2O$ and the UV spectrum of this solution was examined (Figure 3-11) to determine whether this extracted fraction was responsible for the observed UV spectrum of the QBHS/glucose culture medium.

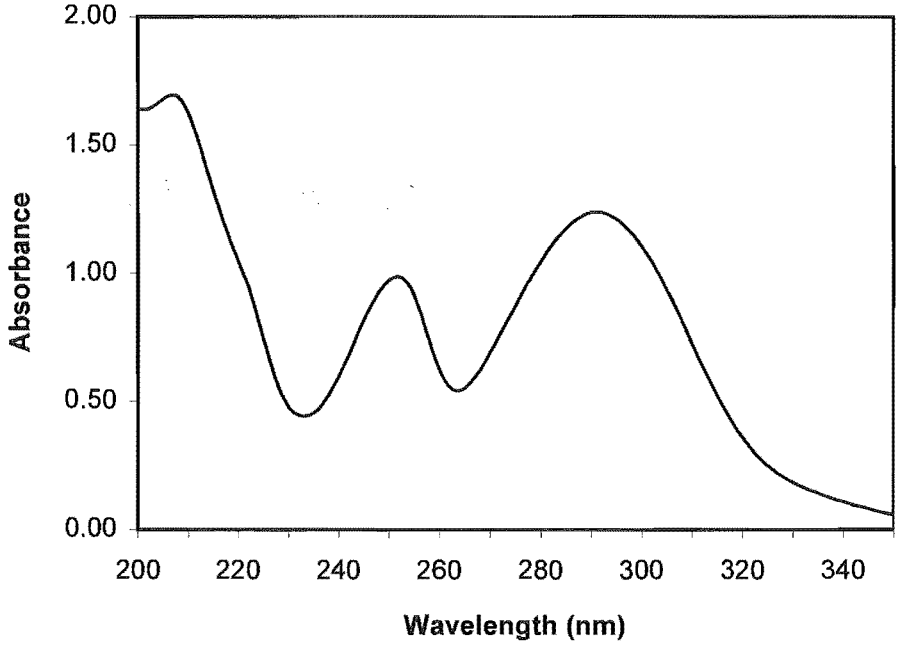


**Figure 3-11. UV spectrum of ethyl acetate soluble material extracted from QP100-8 QBHS/glucose medium.**

As can be observed from Figure 3-11, the ethyl acetate fraction contains the UV active component(s) responsible for the observed spectrum in QBHS/glucose (Figure 3-10). To determine whether a single compound was responsible for the observed UV spectrum, the composition of the ethyl acetate extracted product was examined by HPLC. The chromatogram (Figure 3-12) is consistent with the UV active material being composed of a single compound.

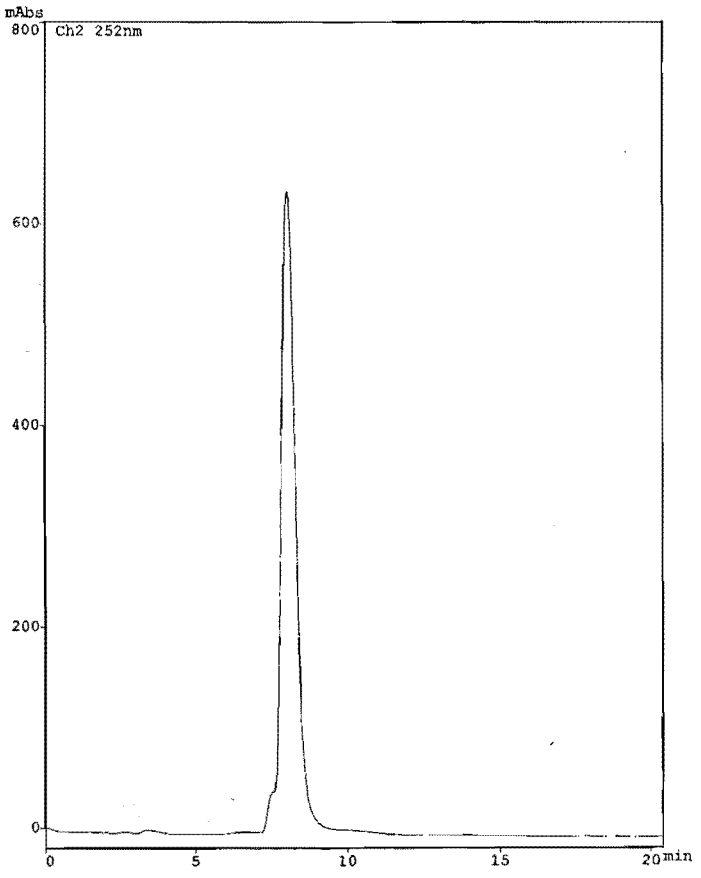


**Figure 3-12. HPLC chromatogram on a C18 column of ethyl acetate-soluble fraction of QP100-8 QBHS/glucose medium.** Analysis performed with 60:40 (v/v) water-methanol mobile phase. Absorbance measured at 252 nm.

To identify this ethyl acetate soluble fraction, proton and carbon NMR analyses were performed. The compound was first purified by the selective precipitation of impurities. The fraction of this material was readily soluble in both ethanol and chloroform with spectrophotoscopic properties expected of 8-hydroxycoumarin: $^{1}$H NMR $\delta$ ($CDCl_3$, 300MHz) 6.41 (1H, d,*J* 9.8 Hz), 7.01 (1H, t,*J* 4.9 Hz), 7.15(2H, d,*J* 4.9 Hz), 7.70 (1H, d,*J* 9.8 Hz) (Figure 3-13); $^{13}$C NMR $\delta$ ($CDCl_3$, 75 MHz) 108.19 (C), 116.41 (CH), 118.13(CH), 119.11 (CH),124.87 (CH), 141.76 (C), 143.42 (C), 144.07 (CH), 159.74 (C) (Cussans and Huckerby 1975).

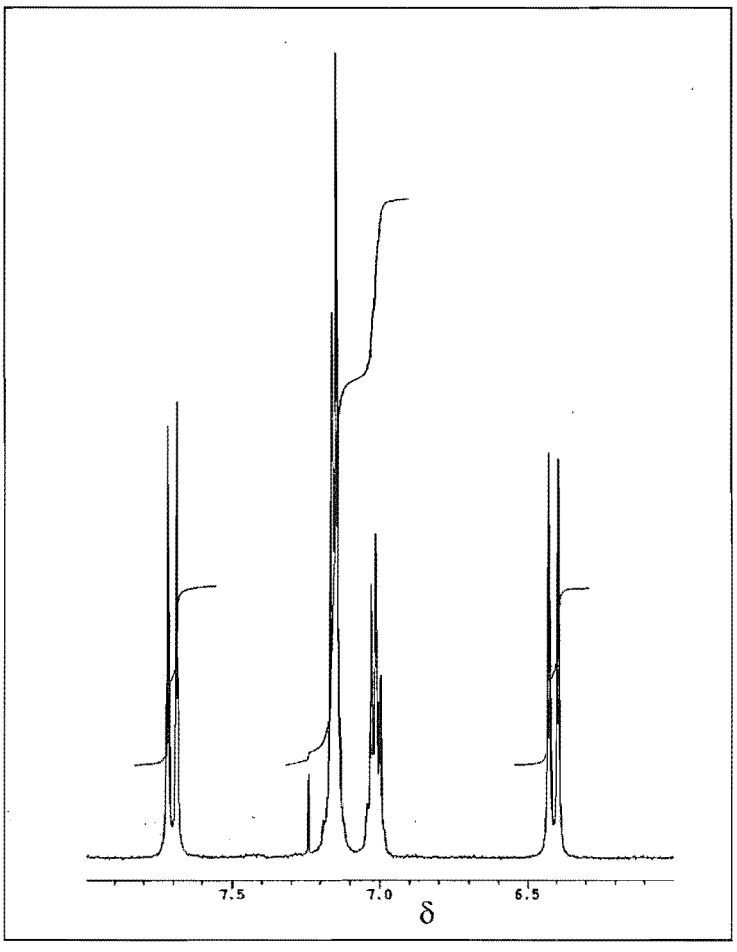


**Figure 3-13. Proton NMR of ethyl acetate soluble compound extracted from QP100-8 QBHS/glucose medium following selective precipitation in both ethanol and chloroform.** Sample run in $CDCl_3$ at 300 MHz.

### 3.5.3 Southern hybridisation analysis of the QP100 mutants

To determine whether the mutant phenotypes of the QP100 mutants had arisen as a result of single transposon insertion events, a Southern hybridisation analysis of the mutants' DNA was performed using Tn5-B21 DNA as a probe. *Bgl*II was the restriction endonuclease selected for fragmenting the QP100 mutant genomic DNA in this experiment, as there is no site for this enzyme within the transposon. *Bam*HI was also selected as there is only a single site for this enzyme 68 bases from the left end of the transposon (Figure 3-8),

Total genomic DNA was isolated from each of the 5 QP100 mutants (QP100-1, -6, -7, -8 & -9) and each sample was treated with *Bam*HI and *Bgl*II separately.

The fragmented DNA was analysed by electrophoresis on the large format apparatus (0.7% w/v agarose, 17.5 hr at 30V). This DNA was then transferred and fixed to a nylon membrane. The membrane was analysed in a Southern analysis using Tn*5*-B21 DNA labelled with [$\alpha$-$^{32}$P]dCTP as a probe, the result of autoradiography can be seen in (Figure 3-14). Each mutant DNA sample provided a single signal which is consistent with a single transposon insertion. As expected, no signal was observed for QP100.

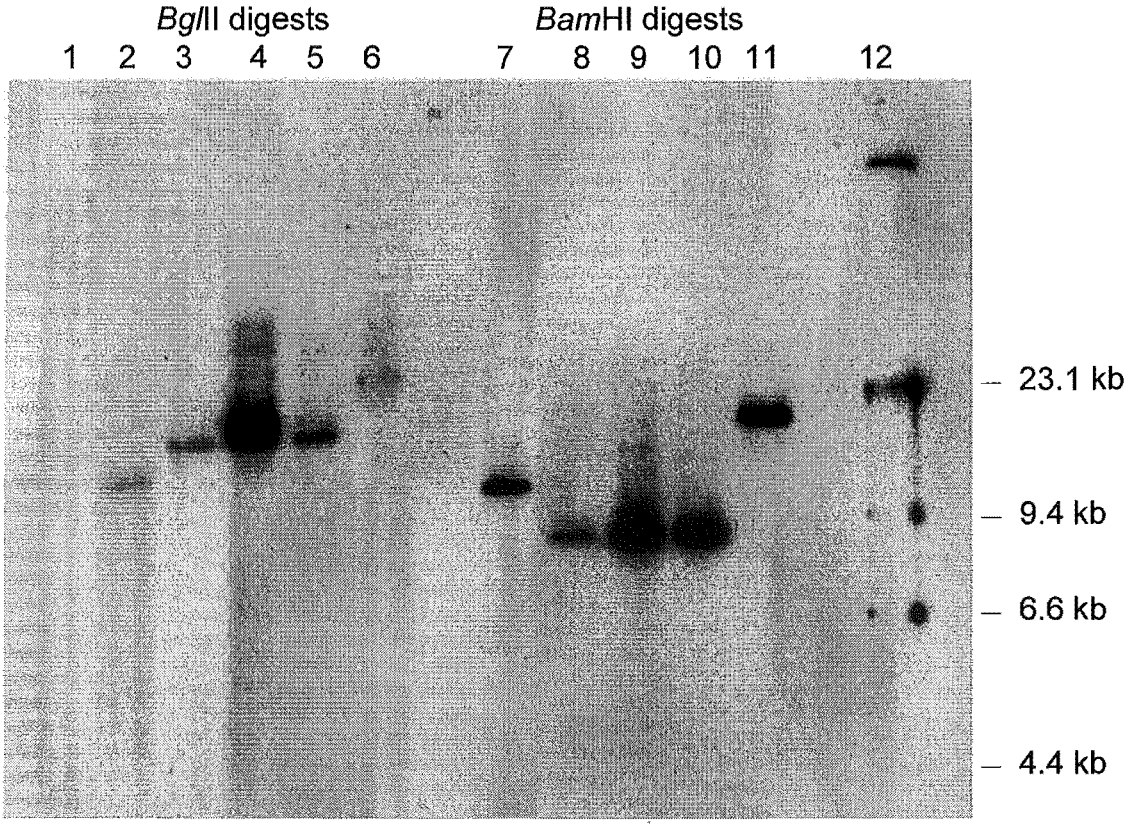


**Figure 3-14. Southern Hybridisation autoradiogram of Tn*5*-B21 induced Qln$^-$ mutants in QP100 using the probe pSUP102::Tn*5*-B21.** Lanes are as follows: 1, QP100; 2, QP100-1; 3, QP100-6; 4, QP100-7; 5, QP100-8; 6, QP100-9 (All digested with *Bgl*II); 7, QP100-1; 8, QP100-6; 9, QP100-7; 10, QP100-8; 11, QP100-9 (All digested with *Bam*HI); 12, $\lambda$ DNA digested with *Hin*dIII.

From Figure 3-14 it can be concluded that Tn*5*-B21 is located in a 9kb *Bam*HI and a 17 kb *Bgl*II fragment in QP100-6, -7 and -8. QP100-1 and QP100-9 both display different insertion patterns. QP100-1 harbours an insertion in a 12 kb

*Bam*HI and a 12 kb *Bgl*II fragment. In QP100-9 the transposon is inserted in a 20 kb *Bam*HI and a 23 kb *Bgl*II fragment.

## 3.6 ISOLATION AND CLONING OF THE QP100-8 *Bgl*II FRAGMENT CONTAINING Tn*5*-B21

The 17 kb *Bgl*II fragment was cloned. It was composed of approximately 8.5 kb of QP100 DNA adjacent to the transposon. The medium copy number plasmid, pACYC184, was selected as the vector to clone this fragment due to its relative stability with larger inserts.

Total genomic DNA was isolated from QP100-8 and was cut with *Bgl*II then extracted with phenol:chloroform, precipitated and dissolved in $dH_2O$. The vector, pACYC184, was first cut with *Bam*HI and following a phenol:chloroform extraction was dephosphorylated, the DNA was precipitated and the final DNA pellet was dissolved in $dH_2O$. The insert (800 ng) was ligated to 100 ng of the vector and this DNA was used to transform DH5$\alpha$ cells. Following elaboration of the transformants, aliquots of these cells were spread onto LB agar plates amended with Tc/Cm and X-gal/IPTG. An aliquot of these transformants was also plated on LB plates containing Cm only and was used to determine ligation efficiency. A dephosphorylation control (dephosphorylated pACYC184 treated with ligase) was also plated onto an LB Tc/Cm agar plate. A total 29 white (these colonies were discarded as they are probably self ligated vectors) and 1 blue transformant were collected on the 3 Tc/Cm plates. Only 3 colonies were seen on the dephosphorylation control plate and approximately 750 colonies on the plate supplemented with Cm. These results suggested both efficient dephosphorylation and ligation.

### 3.6.1 Restriction mapping of pNHQ8

The single blue $Cm^R/Tc^R$ colony was selected because this clone had the expected phenotype of an active *lac*Z gene and $Tc^R$ determinant provided from Tn*5*-B21. This clone was replicated in LB with Cm/Tc and plasmid DNA was

isolated using the alkali lysis minipreparation. The DNA was analysed on a minigel following cutting with *Bam*HI. This produced 2 bands, one of which was approximately 9 kb pairs which was the expected size based on the results from Figure 3-14 and a second band of approximately 12 kb. This recombinant plasmid was designated pNHQ8 and digested with several restriction enzymes (Figure 3-15) to produce a physical restriction map.

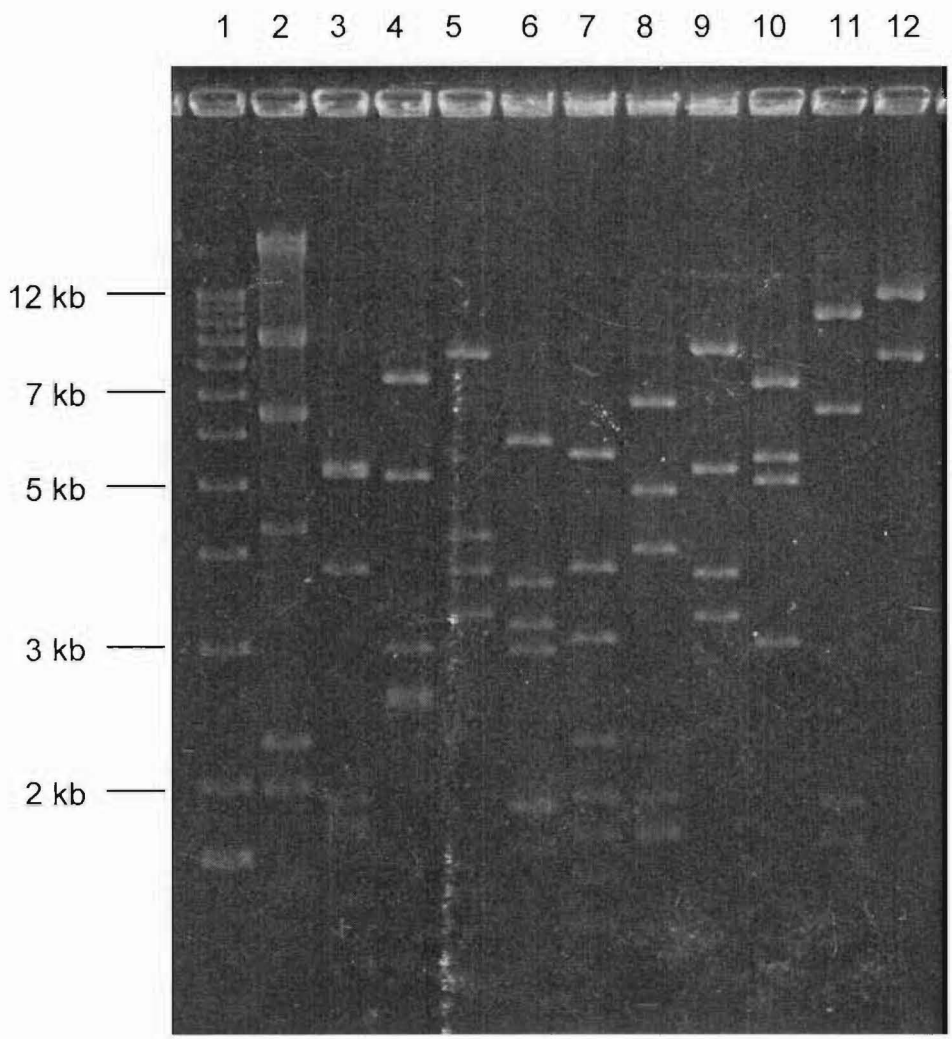


**Figure 3-15. Restriction digests of pNHQ8.** Lanes as follows: 1, BRL 1kb standard; 2, λ *Hin*dIII; 3, *Eco*RV/*Hin*dIII; 4, *Bam*HI/*Eco*RI; 5, *Bam*HI/*Eco*RV; 6, *Eco*RI/*Eco*RV; 7, *Hin*dIII/*Eco*RI; 8, *Hin*dIII/*Bam*HI; 9, *Eco*RV; 10, *Eco*RI; 11, *Hin*dIII; 12, *Bam*HI.

This gel was blotted onto a nylon membrane and analysed by Southern analysis with pACYC184 (labelled with [α-$^{32}$P]dCTP) as a probe, the autoradiogram appears in Figure 3-16.

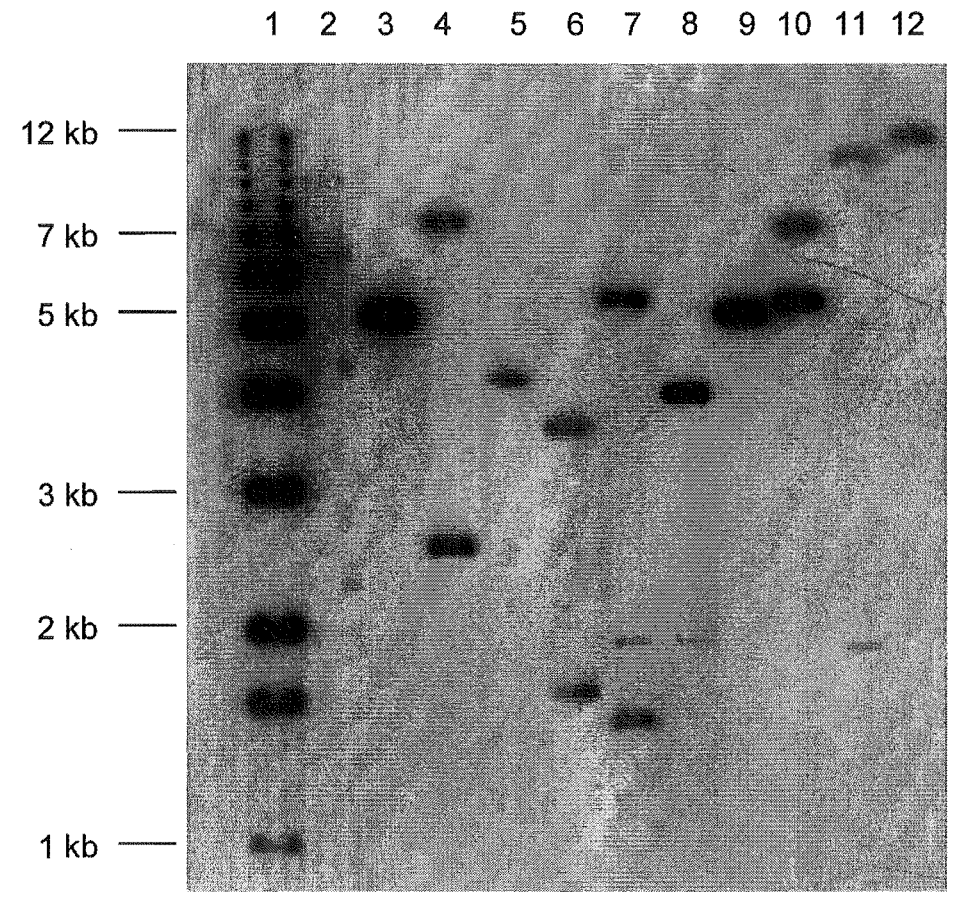


**Figure 3-16. Autoradiogram of restriction digests of pNHQ8 with pACYC184 (labelled with [α-$^{32}$P]dCTP) as the probe.** Lanes as follows: 1, BRL 1kb standard; 2, λ *Hin*dIII; 3, *Eco*RV/*Hin*dIII; 4, *Bam*HI/*Eco*RI; 5, *Bam*HI/*Eco*RV; 6, *Eco*RI/*Eco*RV; 7, *Hin*dIII/*Eco*RI; 8, *Hin*dIII/*Bam*HI; 9, *Eco*RV; 10, *Eco*RI; 11, *Hin*dIII; 12, *Bam*HI.

This autoradiogram enabled the fragments containing portions of pACYC184 to be determined and allowed the restriction map to be completed (Figure 3-17). A linear restriction map of pNHQ8 complete with fragment sizes appears in Appendix III.

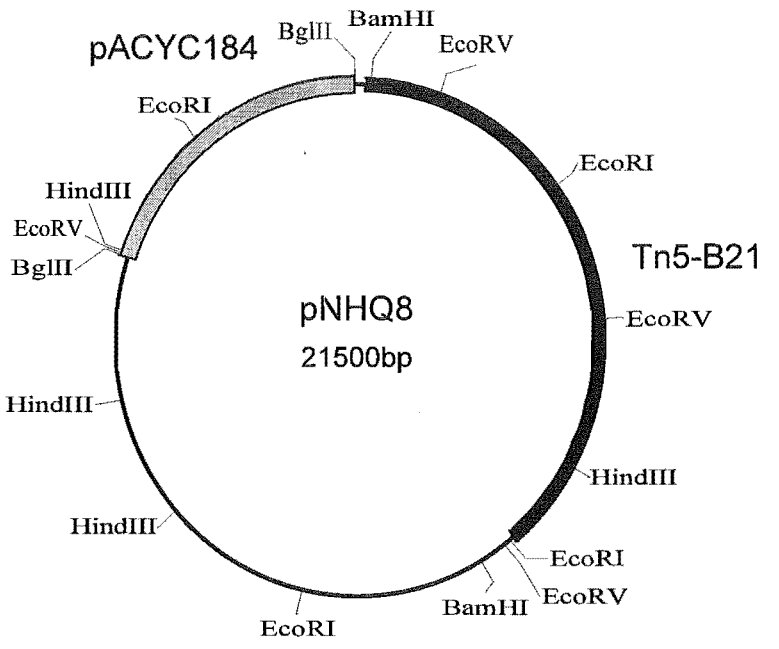


**Figure 3-17. Circular restriction map of pNHQ8.** *Bgl*II sites are shown to delineate the QP100-8 DNA insertion in pACYC184, but no longer exist due to ligation of the compatible ends into the *Bam*HI site in the vector. Tn*5*-B21 DNA appears as a thick black line, pACYC184 as a thick grey line and DNA from QP100-8 as a narrow black line. Fragment sizes are listed in Appendix III.

## 3.7 SUBCLONING PORTIONS OF pNHQ8 INTO pUC19

To facilitate sequencing of the QP100 DNA adjacent to the transposon insertion, and to aid with the manipulation of the fragments of pNHQ8, various restriction fragments were subcloned into pUC19 (Figure 3-18).

Derivatives of pNHQ8 were created as follows. pNHQ81 was produced by digesting pNHQ8 with *Bam*HI and ligating this mixture with similarly treated pUC19 (but also dephosphorylated), this reaction mix was used to transform DH5α. $Ap^R$ transformants were selected and screened for β-galactosidase activity on LB Tc/Ap X-gal/IPTG plates. 5 white colonies (β-galactosidase activity from Tn*5*-B21 was destroyed by cleavage with *Bam*HI therefore insertional inactivation of the *lacZ* gene in pUC19 could be utilised as a means

of selection) were selected at random. Plasmid DNA of the 5 replicated clones was examined by electrophoresis following treatment with *Bam*HI. A clone displaying DNA fragments of 9 kb and 2.7 kb was selected and its plasmid was designated pNHQ81.

A construct with most of the transposon DNA removed from pNHQ81 was produced by cutting this plasmid with *Hin*dIII and *Bam*HI. Following electrophoresis the 1.7 kb band was excised. The DNA was extracted from this slice of agarose and ligated to pUC19 cut with both *Hin*dIII and *Bam*HI. This DNA was used to transform DH5α, 5 white $Ap^R$ colonies where selected from LB/Ap/X-gal/IPTG plates and their plasmid DNA extracted. This plasmid DNA was examined by restriction digest with *Hin*dIII and *Bam*HI both in combination and singularly followed by electrophoresis. A clone was selected displaying both the 1.7 and 2.7 kb fragments in the double digests and a single fragment of 4.4 kb with a single enzyme treatment. This plasmid was designated pNHQ82.

pNHQ83 was produced in a similar manner to pNHQ82, but the *Hin*dIII digest was replaced with an *Eco*RI digest. The resulting 0.5 kb fragment was ligated into pUC19 and the appropriate clone was selected as with pNHQ82 but with the appropriate size restriction fragments (ie: 0.5 kb insert, 2.7 kb vector and 3.2 kb total size).

The subclone, pNHQ84, was produced by digestion of pNHQ8 with *Eco*RI. The 3 kb fragment ligated into pUC19 previously treated with *Eco*RI and dephosphorylated. Following transformation of DH5α with the recombinant plasmids, several white $Ap^R$ transformants were chosen and their plasmid DNA was checked by restriction endonuclease analysis and AGE, a single clone containing the appropriate size fragment was selected.

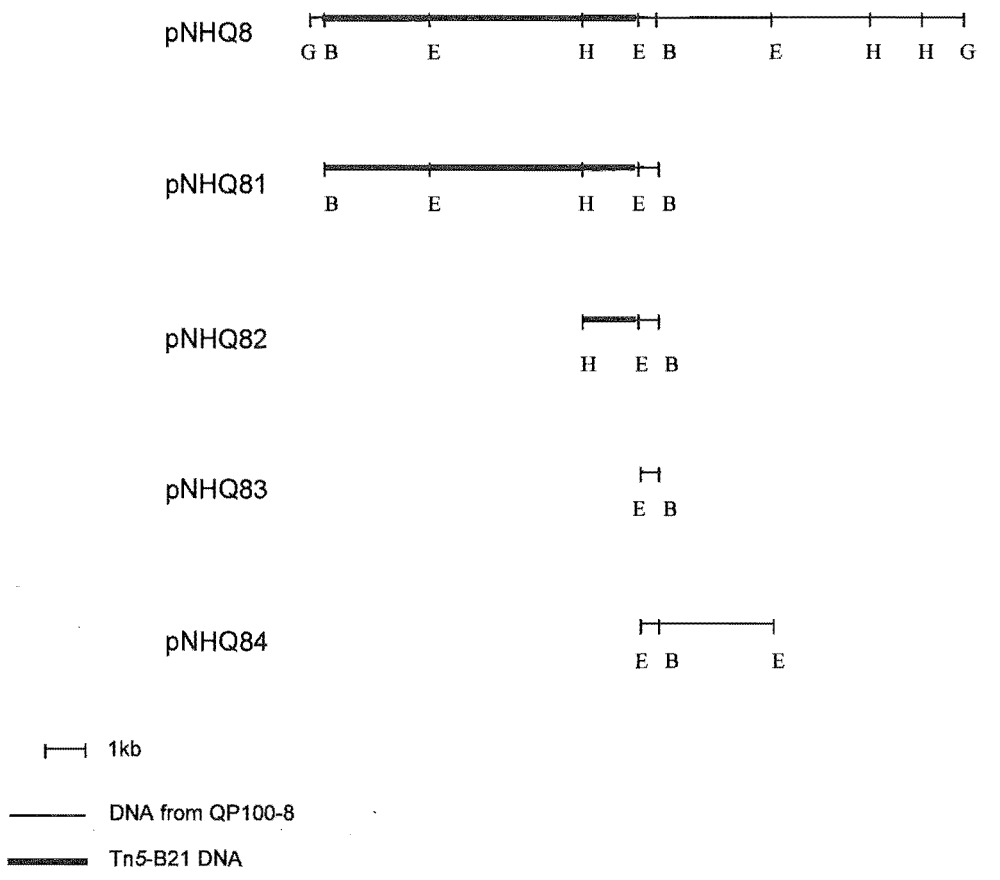


**Figure 3-18. Restriction maps of inserts of QP100-8 DNA in the pNHQ8 series of plasmids.** The 17 kb fragment of DNA harbouring the Tn*5*-B21 transposon was isolated from QP100-8 and ligated into pACYC184. The fragments in pNHQ81 and pNHQ84 where derived from pNHQ8, pNHQ82 and pNHQ83 contained inserts from pNHQ81 and all were ligated into pUC19. Restriction enzymes: B, *Bam*HI; E, *Eco*RI; G, *Bgl*II and H, *Hin*dIII.

## 3.8 CLONING THE wt SEQUENCE FROM QP100

It was desirable to clone the intact sequence of DNA from QP100 which in QP100-8 is interrupted by the transposon. This DNA sequence, which could be functionally active, might then complement the QP100 mutants. The clone would also provide DNA sequence data to the left (as it is seen in Figure 3-18) of the transposon. *Bam*HI and *Hin*dIII were chosen as they provided 0.5 kb and 5.5 kb of DNA respectively, to the right of the transposon insertion in QP100-8 (as it is seen in Figure 3-18). To determine the size of these corresponding fragments in QP100, the following experiment was performed.

QP100 DNA was fragmented with *Hin*dIII and *Bam*HI separately. The DNA was analysed by AGE and immobilised onto a nylon membrane. This membrane was probed with pNHQ82 (labelled with [$\alpha$-$^{32}$P]dCTP) to determine the size of the fragment giving a positive signal, and therefore the size of the equivalent fragment in QP100 which had the transposon inserted in QP100-8. The autoradiograms for the *Bam*HI and *Hin*dIII digests of QP100 can be seen in Figure 3-19.

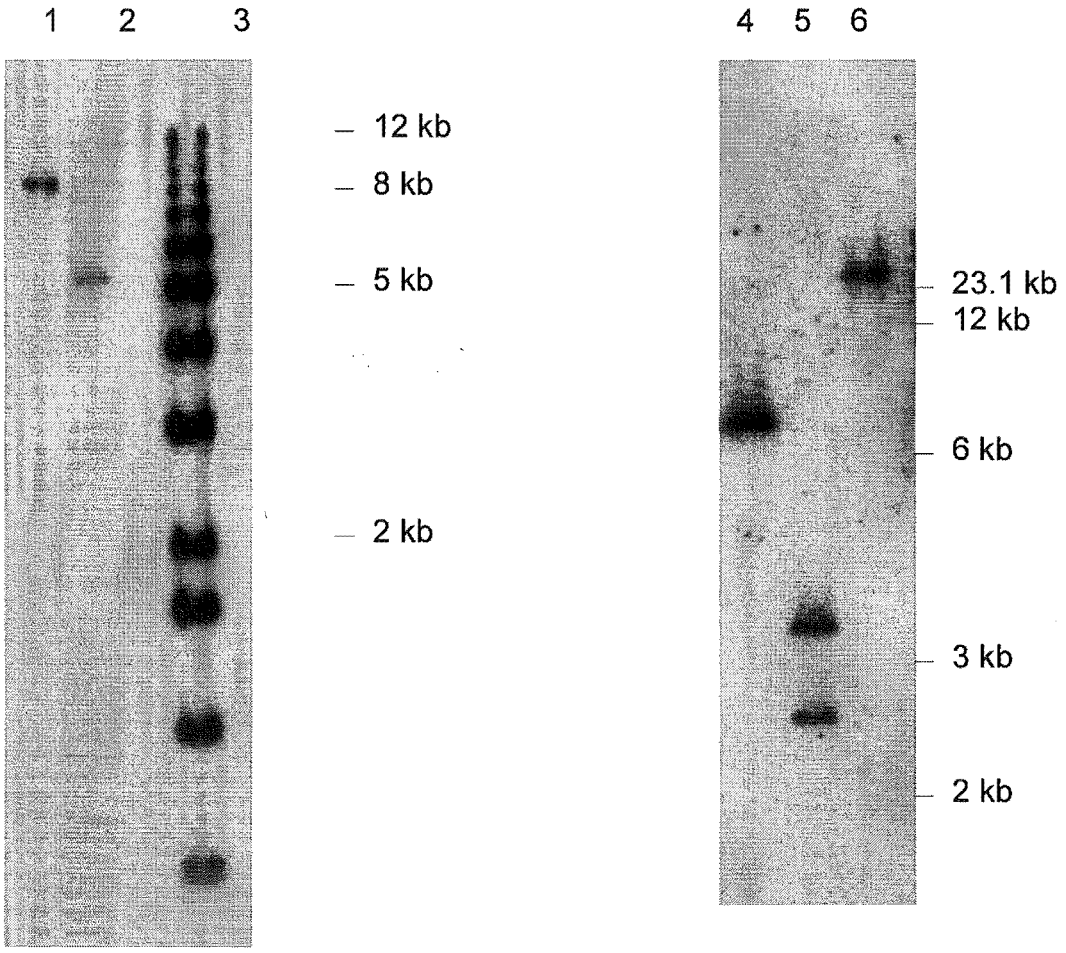


**Figure 3-19. Separate autoradiograms of QP100 and QP100-8 treated with *Bam*HI (left autoradiogram) and *Hin*dIII (right autoradiogram) both have been probed with pNHQ82 (labelled with [$\alpha$-$^{32}$P]dCTP).** Lanes are as follows: 1, QP100-8; 2, QP100 (both treated with *Bam*HI); 3, BRL 1 kb standard; 4, QP100-8 (*Hin*dIII treated); 5, QP100-8 (*Hin*dIII/*Eco*RI treated); 6, QP100 (*Hin*dIII treated).

*Bam*HI was selected for cloning this uninterrupted fragment in QP100 as the fragment displaying the positive signal in Figure 3-20 was approximately 5 kb.

This was considered to be a more manageable size for cloning than the >23 kb fragment produced by *Hin*dIII. The procedure for cloning the 5 kb *Bam*HI from QP100 is outlined diagrammatically in Figure 3-20.

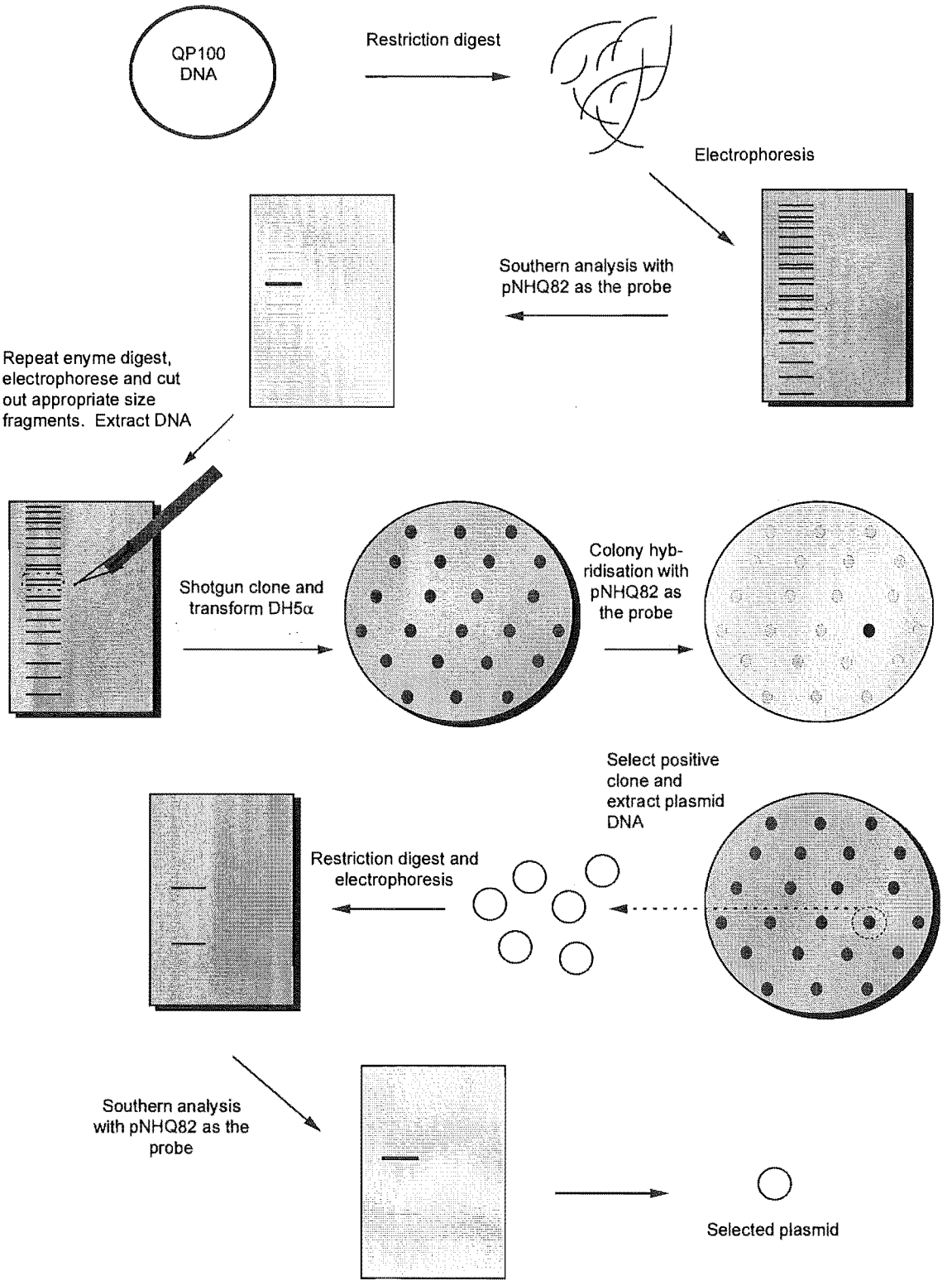


**Figure 3-20. Diagrammatic representation of the construction of pC1 in pUC19 from total genomic QP100 DNA.**

QP100 DNA was again cut with *Bam*HI and electrophoresed as before, a slice of agarose containing the appropriate size range calculated from Figure 3-19 (4-6 kb, approximately) of DNA fragments was excised from the gel. This DNA was extracted from the agarose (GeneClean®) and ligated to *Bam*HI digested and dephosphorylated pUC19 DNA. These recombinant plasmids were used to transform DH5α cells which were tested for β-galactosidase activity. The white/$Ap^R$ transformants were selected and 1000 of these colonies were transferred, in duplicate, to fresh LB/Ap plates and allowed to grow o/n. From one set of the duplicate plates, the colonies were transferred to nylon membranes and following DNA liberation and immobilisation, these membranes were probed with the 1.7 kb *Bam*HI/*Hin*dIII fragment extracted from pNHQ82 and labelled with [α-$^{32}$P]dCTP. The resulting image from the Storm™ 840 phosphorimager appears in Figure 3-21.

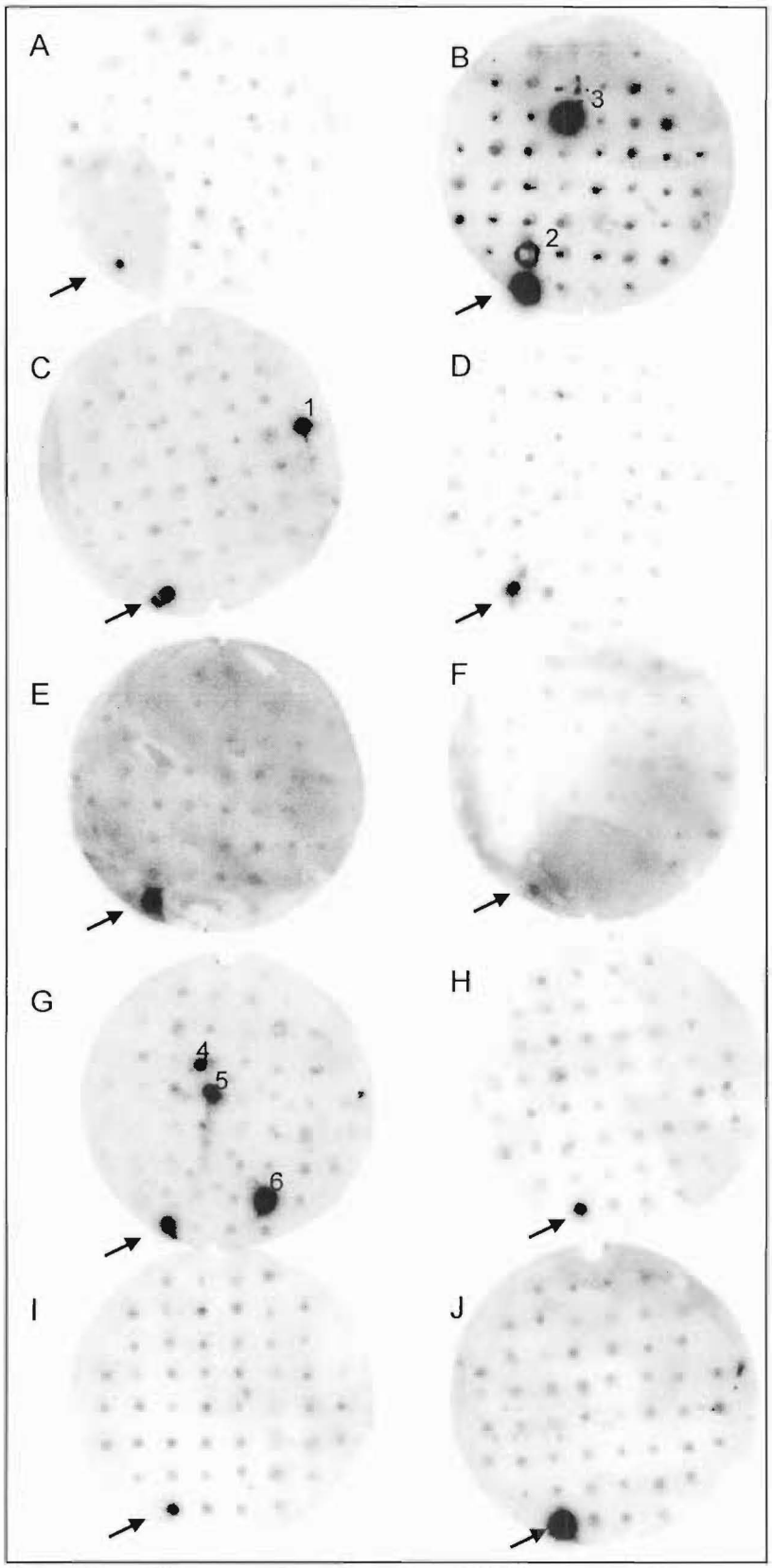


**Figure 3-21. ImageQuant™ image from the Storm™ 840 phosphorimager autoradiogram of colony hybridisation probed with the 1.7 kb *Bam*HI/*Hin*dIII fragment extracted from pNHQ82 labelled with [α-$^{32}$P]dCTP.** Arrows point to positive control colonies harbouring pNHQ82, colonies adjudged to return positive signals are numbered. Background has been adjusted to give a visible signal to all colonies to allow for ease of location on the appropriate rescue plates.

Positive signals were compared to that of the positive controls on each membrane (DH5α, pNHQ82, marked with arrows in Figure 3-21). Six colonies were chosen based on this comparison (numbered in Figure 3-21). The corresponding colonies were removed from the duplicate plates which represented those returning positive signals on the colony hybridisation. Plasmid DNA was extracted from these clones and cut with *Bam*HI and the DNA was electrophoresed on a minigel apparatus (Figure 3-22) before being transferred to a nylon membrane.

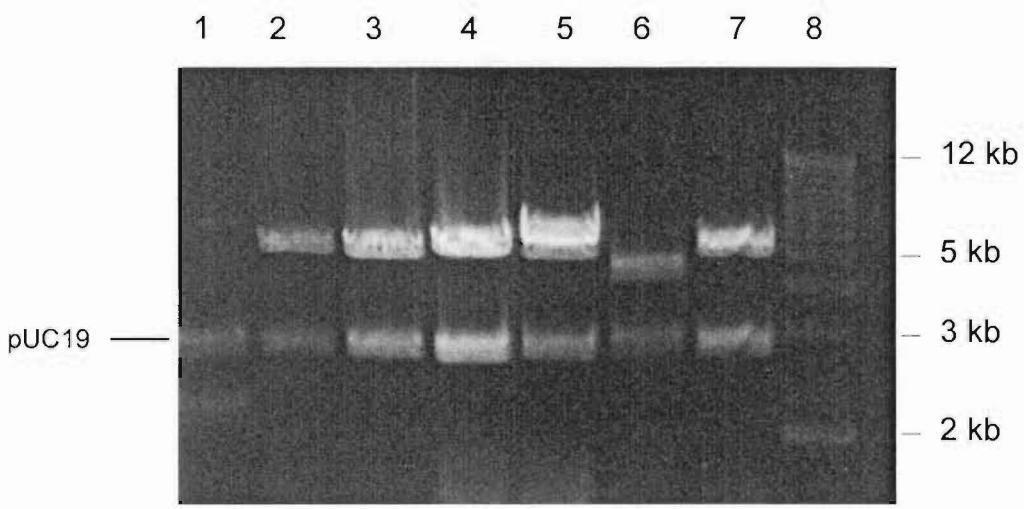


**Figure 3-22. *Bam*HI restriction digests of pUC19 clones containing *Bam*HI fragments from QP100.** Lanes are as follows: 1, pNHQ82 (*Bam*HI/*Hin*dIII treated); 2, pG6; 3, pG5; 4, pG4; 5, pB3; 6, pB2; 7, pC1 (all treated with *Bam*HI); 8, BRL 1kb standard.

The nylon membrane was probed with the 1.7 kb *Bam*HI/*Hin*dIII fragment extracted from pNHQ82 labelled with [α-$^{32}$P]dCTP and the result was analysed on the Storm™ 840 (Figure 3-23).

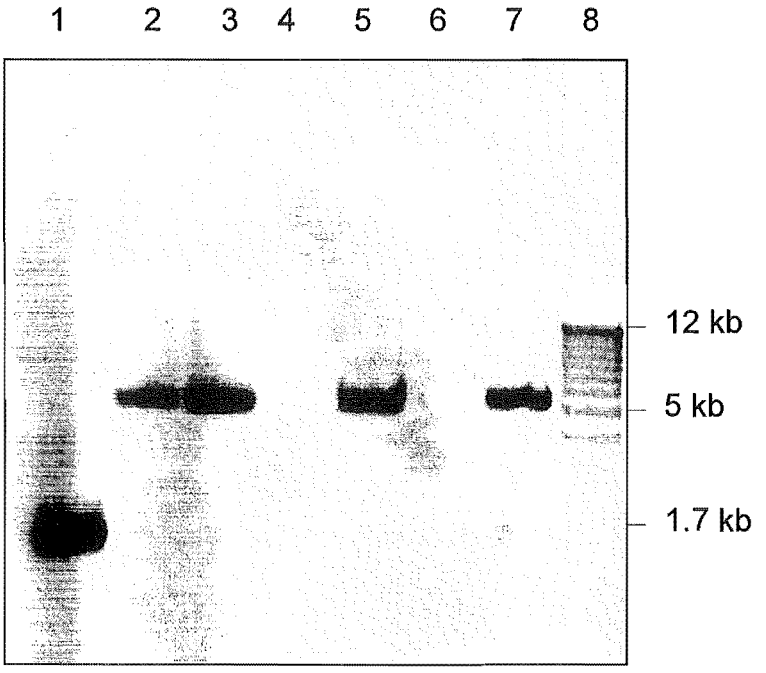


**Figure 3-23. ImageQuant™ image from the Storm™ 840 phosphorimager autoradiogram of the blotted gel in Figure 3-22, the 1.7 kb *Bam*HI/*Hin*dIII insert from pNHQ82 was used as a probe (labelled with [α-$^{32}$P]dCTP).** Lanes are as follows: 1, pNHQ82 (*Bam*HI/*Hin*dIII treated); 2, pG6; 3, pG5; 4, pG4; 5, pB3; 6, pB2; 7, pC1 (all treated with *Bam*HI); 8, BRL 1kb standard.

The plasmids pC1, pB3, pG5 and pG6 all returned positive signals. pB2 and pG4 provided no signal and were discarded. To check for double insertions pC1, pB3, pG5 and pG6 were digested with *Eco*RI and *Bgl*II/*Bam*HI. The result of AGE is seen in Figure 3-24. As pB3 appears to have a double insertion, it was not studied further.

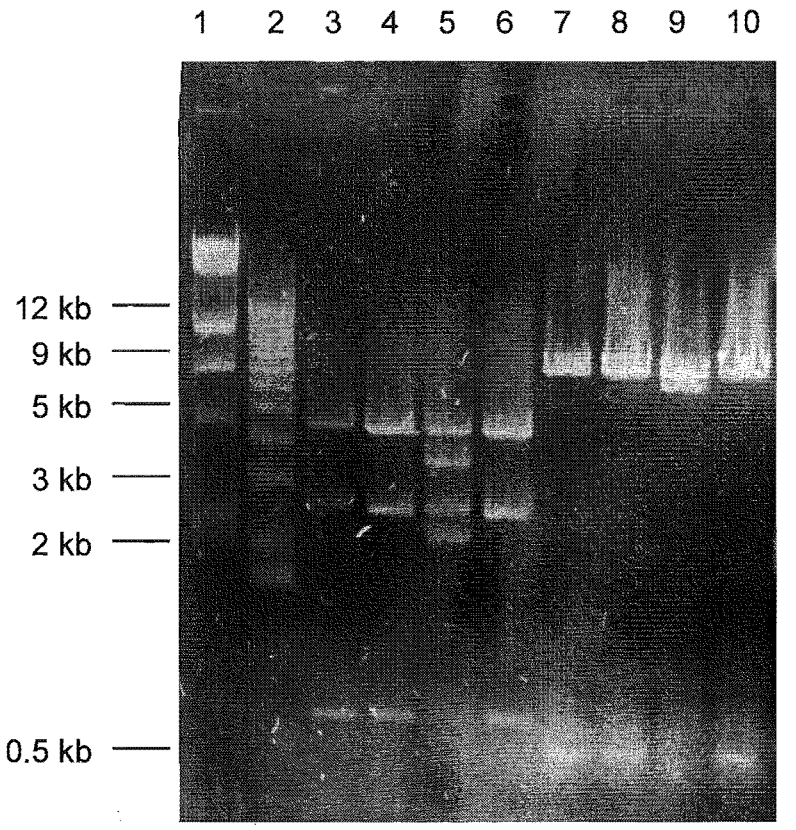


**Figure 3-24. Restriction digestions of pC1, pB3, pG5 and pG6.** Lanes are as follows: 1, λ *Hin*dIII; 2, BRL 1 kb standard; 3, pG6; 4, pG5; 5, pB3; 6, pC1 (all treated with *Bam*HI/*Bgl*II); 7, pG6; 8, pG5; 9, pB3; 10, pC1 (all treated with *Eco*RI).

Therefore, these results are consistent with pC1, pG5 and pG6 harbouring the same appropriate *Bam*HI fragment from QP100 (the restriction map appears in Figure 3-25).

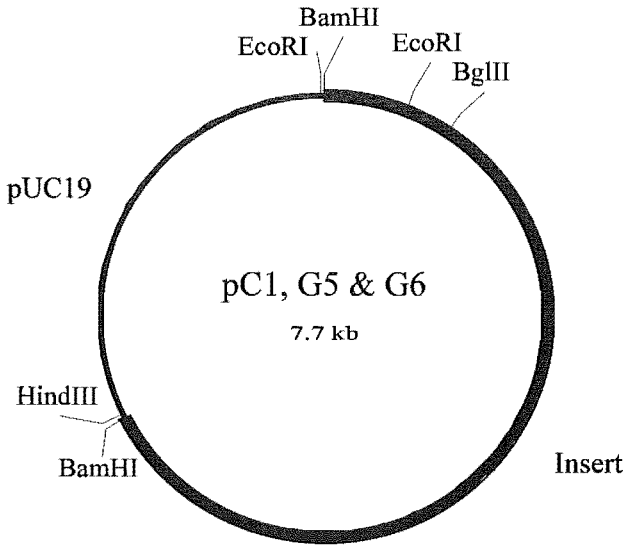


**Figure 3-25. Restriction map of pC1, pG5 and pG6.** Insert DNA is from QP100.

The plasmid pC1 was selected for further study, pG5 and pG6 were also stored to provide a backup to pC1, as possible mutations acquired during the cloning process may interfere with future complementation experiments.

## 3.9 COMPLEMENTATION OF QP100 MUTANTS

Before complementation experiments could be performed, the 5 kb *Bam*HI fragment from pC1 had to be ligated into a vector capable of replication in *P. aeruginosa*. As less than 500 bases of this DNA fragment represented DNA to the right of the transposon insertion as it is seen in Figure 3-18 (shown in pNHQ8), it was presumed that a further sequence of DNA would need to be added to extend the size of this fragment. DNA from pNHQ84 (the 3 kb *Eco*RI fragment) would be added to this *Bam*HI fragment to produce a clone containing a large enough sequence (total of 7.5 kb, approximately) to complement QP100-8 (this hypothetical construct is shown in Figure 3-26).

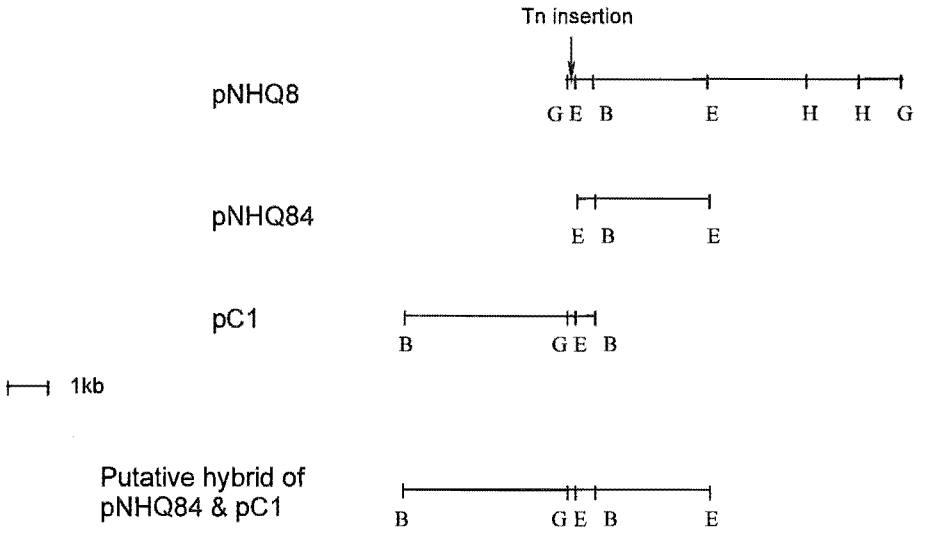


**Figure 3-26. Diagram showing the alignment of the restriction fragments from pNHQ8, pNHQ84 and pC1.** The putative hybrid of pNHQ84 and pC1 is shown, this DNA sequence will be assembled with the aim of reconstructing the sequence interrupted by the transposon in QP100-8 (depicted in pNHQ8 above). Restriction enzymes: B, *Bam*HI; E, *Eco*RI; G, *Bgl*II and H, *Hin*dIII.

### 3.9.1 Assembly of QP100 DNA fragments in pRO1614

The plasmid pRO1614 was chosen as the vector for assembly of a clone for complementation experiments in QP100-8. This plasmid was constructed from pBR322, which cannot replicate in *P. aeruginosa*, and a *Pst*I fragment, believed to be derived from RP1 (Olsen et al. 1982). This *Pst*I fragment appears to allow the vector to replicate in non-enteric hosts such as *P. aeruginosa*. pRO1614 confers resistance to carbenicillin and tetracycline, the latter is insertionally inactivated if a fragment of DNA is inserted in the *Bam*HI site which lies within the β-lactamase gene.

Firstly the 4.5 kb *Bam*HI/*Eco*RI fragment from pC1 was added to pRO1614, this would give directional insertion of the fragment into the vector due to the asymmetric ends provided by the two different enzymes. Both pRO1614 and pC1 were cut with *Bam*HI and *Eco*RI and the resulting fragments were separated on a minigel. The 4.5 kb fragment from pC1 was excised and the

DNA purified using GeneClean®, this was ligated to the similarly treated pRO1614. The ligation products were used to transform DH5α and 6 $Ap^R/Tc^S$ transformants were chosen at random. Their plasmid DNA was cut with *Bam*HI and *Eco*RI and the result was examined by AGE. Three of these clones all appear to have the expected restriction fragments and their plasmids were designated pNHQR1, pNHQR2 and pNHQR3 (digests of pNHQR2 and pNHQR3 are seen in Figure 3-27 below).

Further experiments were initially limited to pNHQR2, the 2 other plasmids were stored as a precaution, should there be a problem with pNHQR2, again if mutations acquired during the cloning procedure affected future complementation experiments.

To ensure a greater chance of complementation, a further 3 kb of DNA was added to pNHQR2 from pNHQ84. The 3 kb *Eco*RI fragment from pNHQ84 was liberated and separated on a minigel, the DNA was extracted with GeneClean®, then ligated to *Eco*RI cut and dephosphorylated pNHQR2. DH5α was then transformed with these ligation products and plated on LB/Ap plates. Ten of these $Ap^R$ transformants were screened for the presence of an appropriate size plasmid, 2 of these plasmid digests showed restriction fragments of the appropriate size (Figure 3-27) and these were designated pNHQR4 and pNHQR8.

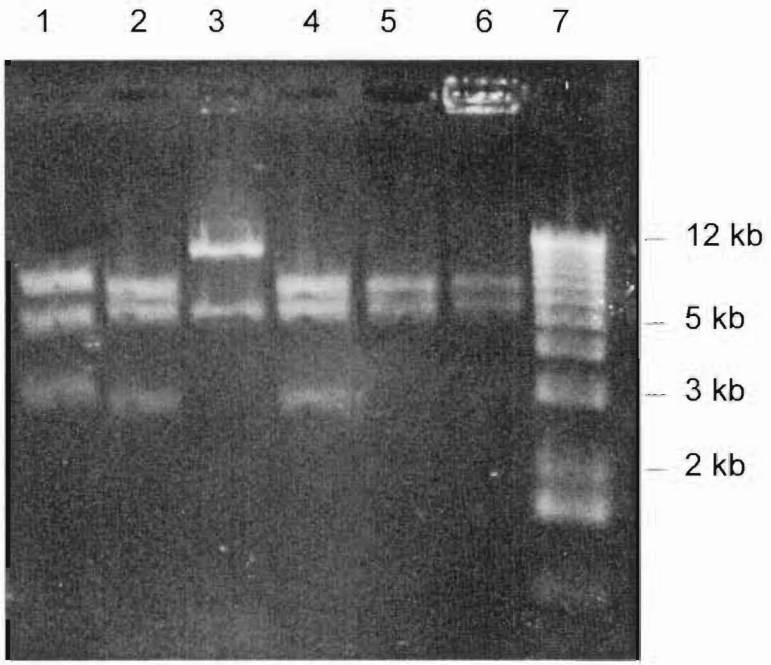


**Figure 3-27. Restriction digests of the pNHQR series of plasmids.** Lanes are as follows: 1, pNHQR8 (*Bam*HI/*Bgl*II); 2, pNHQR8 (*Bam*HI/*Eco*RI); 3, pNHQR4 (*Bam*HI/*Bgl*II); 4, pNHQR4 (*Bam*HI/*Eco*RI); 5, pNHQR3; 6, pNHQR2 (both with *Bam*HI/*Eco*RI).

From this restriction fragment pattern it can be seen pNHQR4 must contain the 3 kb *Eco*RI fragment in the correct orientation (ie: that observed in with pNHQ8) (Figure 3-28), and pNHQR8 contains the *Eco*RI fragment in the reverse orientation.

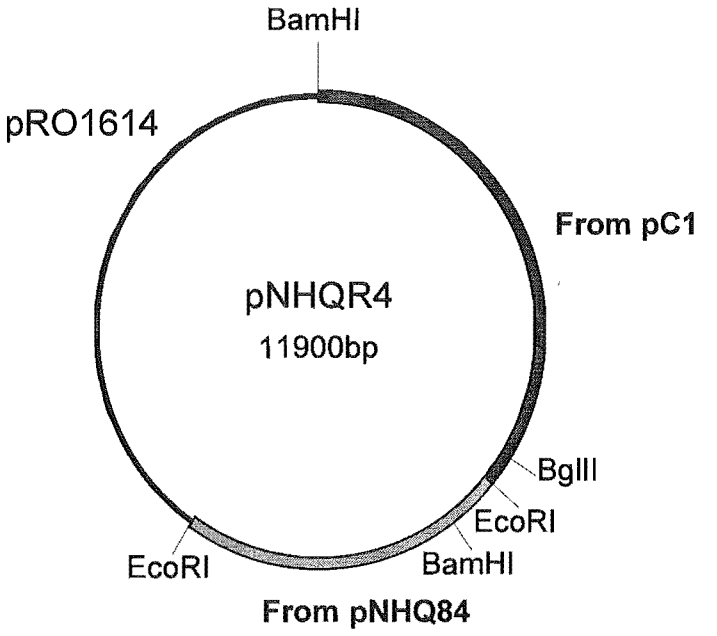


**Figure 3-28. Restriction map of pNHQR4 detailing source of each component.**

### 3.9.2 Complementation of the QP100 mutants

Complementation experiments were performed with the plasmids pNHQR2, pNHQR4 and pNHQR8. These plasmids had been maintained in DH5α for ease of manipulation but could not be used to transform QP100 strains directly as DNA from *E. coli* does not transform QP strains (Table 3-3).

**Table 3-3 Transformation of *P. aeruginosa* with pRO1614 from both *E. coli* and *P. aeruginosa* host backgrounds.**

| pRO1614 DNA isolated from: | Recipient cells[a] | |
|---|---|---|
| | OT684 | QP100 |
| *E. coli* | $1.2\times10^6$ | ND (≤10 transformants/μg) |
| *P. aeruginosa* (OT684) | - | 30 |
| *P. aeruginosa* (QP100) | - | $1.5\times10^4$ |

[a]transformants/μg of pRO1614

The plasmids were first replicated in OT11 before being used to transform QP strains, as DNA from *P. aeruginosa* strains, unlike DNA isolated from *E. coli*, can be used to transform QP strains.

*P. aeruginosa* OT11 was transformed with each of the pNHQR plasmids separately and the resulting transformants were selected on LB/Cb plates. Plasmid DNA was extracted from the $Cb^R$ clones and checked for appropriate size restriction fragments (Figure 3-29) this was necessary as OT11 is capable of homologous recombination and the pNHQR plasmids contain *P. aeruginosa* DNA of unknown homology to OT11. No rearrangement of these plasmids is evident from this minigel as all fragment sizes are those expected of pNHQR4. QP100-8 was then electroporated with pNHQR4 plasmid DNA isolated from OT11 and the $Cb^R/Tc^R$ transformants were selected.

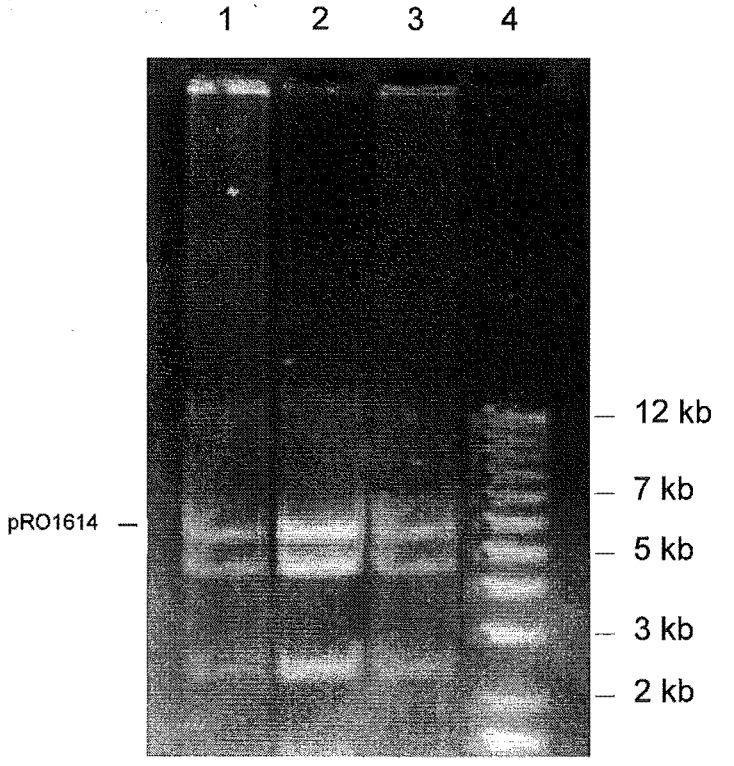


**Figure 3-29. pNHQR4 plasmid DNA isolated from QP100-8, OT11 and DH5α, digested with *Bam*HI/*Eco*RI.** Lanes are as follows: 1, QP100-8; 2, OT11; 3, DH5α; 4, BRL 1kb standard.

QP100-6 and QP100-7 were also transformed with the three pNHQR plasmids, and QP100-8 with pNHQR8 and pNHQR2. The physical maps of the fragments in the pNHQR plasmids tested for complementation in the QP100 mutants are shown in Figure 3-30 compared to QP100-8.

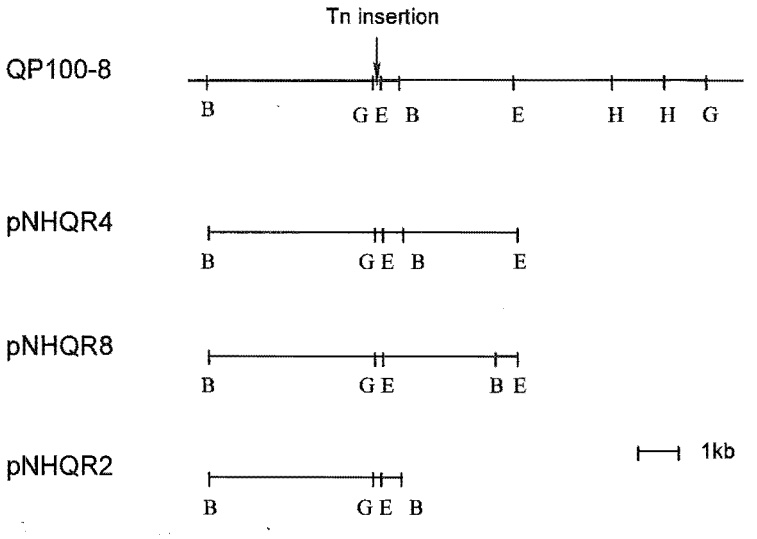


**Figure 3-30. Physical maps of the pNHQR series of plasmids used in complementation experiments with Qln⁻ mutants.** Plasmid maps are shown aligned to their equivalent sequence in QP100-8. Note reverse orientation of the *Eco*RI fragment in pNHQR8. Restriction enzymes: B, *Bam*HI; E, *Eco*RI; G, *Bgl*II and H, *Hin*dIII.

Single colonies of each of the QP100-6, -7, -8 transformants (each transformed with pNHQR2, pNHQR4 and pNHQR8) were patched onto quinoline assay plates. Their growth was examined periodically for 4 days at 30 °C and compared to that of untransformed QP100, OT11, QP100-6, -7 & -8 (Figure 3-31).

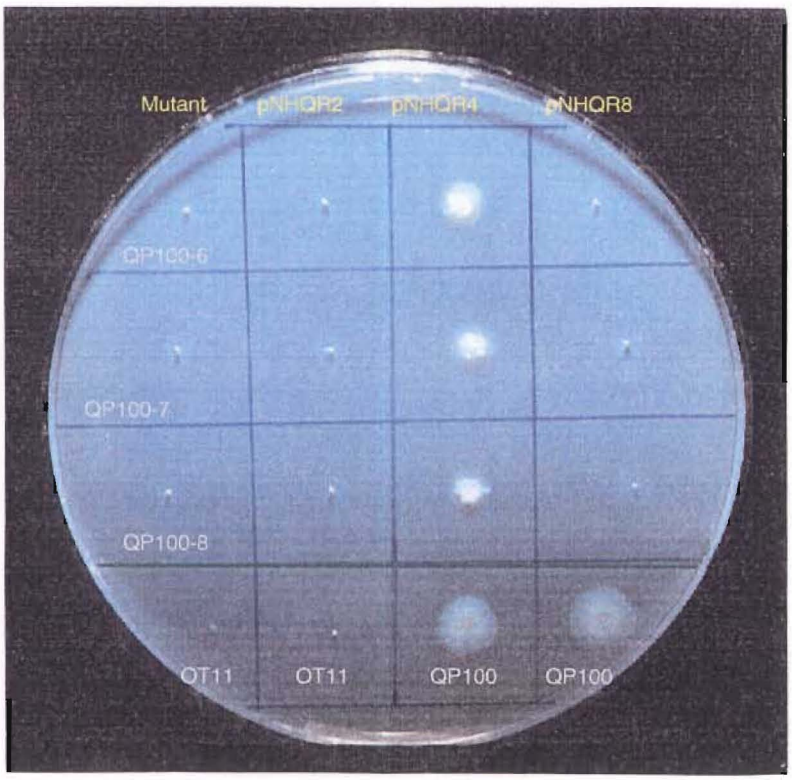


**Figure 3-31. Plate assay of the complemented mutants**. Columns are marked in yellow, rows in grey. Inoculations below the green line are negative (OT11) and positive (QP100) controls.

It can be concluded from Figure 3-31 that pNHQR4 contains a sequence of DNA that returns QP100-6,-7 & -8 to the $Qln^+$ phenotype. The sequence contained within pNHQR2 and pNHQR8 did not revert any of QP100-6, -7, -8 mutants to $Qln^+$.

## 3.10 EXPRESSION OF pNHQR4 IN OT11

The DNA sequence contained in pNHQR4 was shown to complement QP100-6, -7 & -8 to $Qln^+$. To determine whether this plasmid encoded a structural gene expressible in another *P. aeruginosa*, the following experiments were performed. A single colony OT11 pNHQR4 was inoculated into BHS medium supplemented with $NH_4NO_3$, 0.2% (w/v) glucose, proline, leucine, carbenicillin and 8-hydroxycoumarin (isolated from the culture medium of QP100-8 as described in 3.5.2). Similar cultures were set up with OT11 (pRO1614), as a

negative control, and QP100-8 (pNHQR4), as a positive control). The flasks were incubated at 30 °C (with aeration) for 48 hours after which, the cells and precipitated matter were removed by centrifugation. The medium supernatant was extracted with ethyl acetate as described in 2.14.1 and resulting compounds dissolved in $dH_2O$. The UV spectra of diluted aliquots were examined (Figure 3-32).

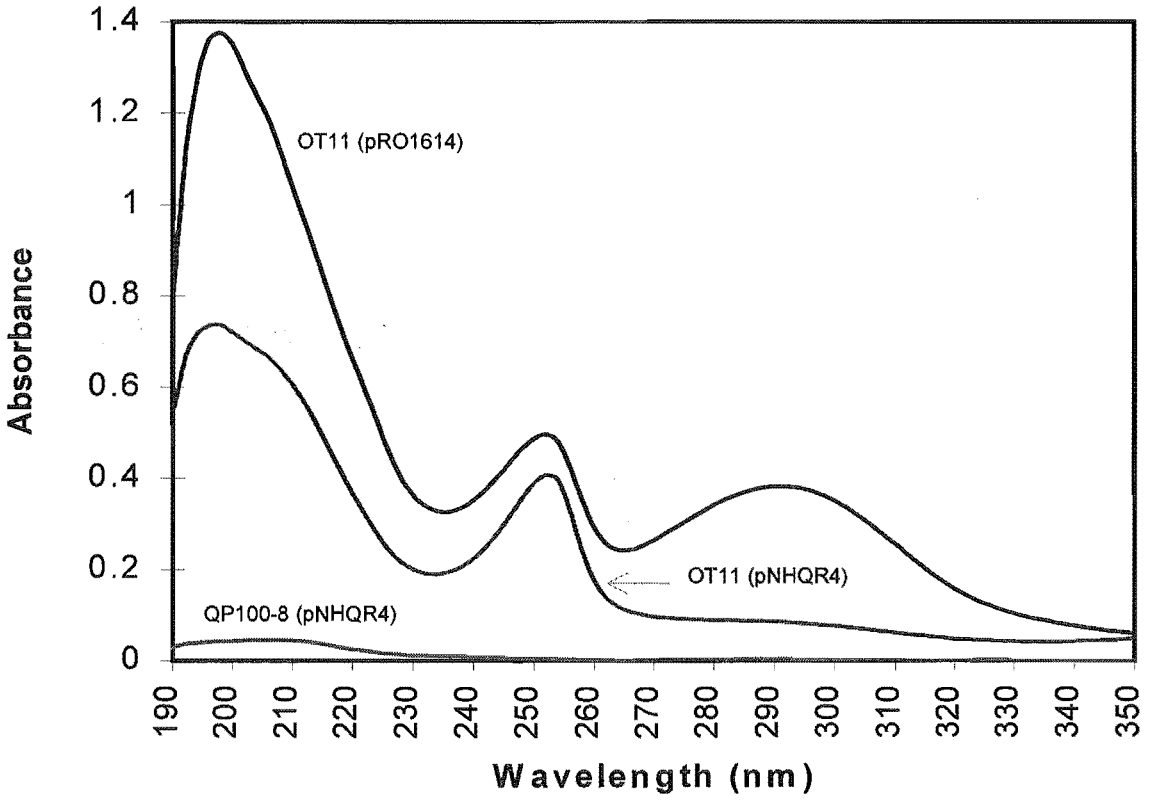


**Figure 3-32. Overlayed spectra of ethyl acetate soluble material extracted from culture medium containing 8-hydroxycoumarin.**

It can be concluded from Figure 3-32 that no detectable 8-hydroxycoumarin remains in the medium of the complemented mutant QP100-8 pNHQR4. The negative control strain, OT11 (pRO1614), cannot degrade 8-hydroxycoumarin and therefore this compound remains in the culture medium as indicated by the UV spectrum being that expected of 8-hydroxycoumarin. The UV spectrum

from the culture medium extract of OT11 pNHQR4 contains a compound that is different from the supplied 8-hydroxycoumarin as this new compound does not have a peak in absorbance at 292 nm as expected with 8-hydroxycoumarin.

To assist with the identification of this putative new compound produced by OT11 pNHQR4 HPLC analyses were performed. Aliquots of the ethyl acetate soluble material extracted from the culture media of OT11 pRO1614 (Figure 3-34) and OT11 pNHQR4 (Figure 3-35) were compared by HPLC to the previously extracted 8-hydroxycoumarin (Figure 3-33).

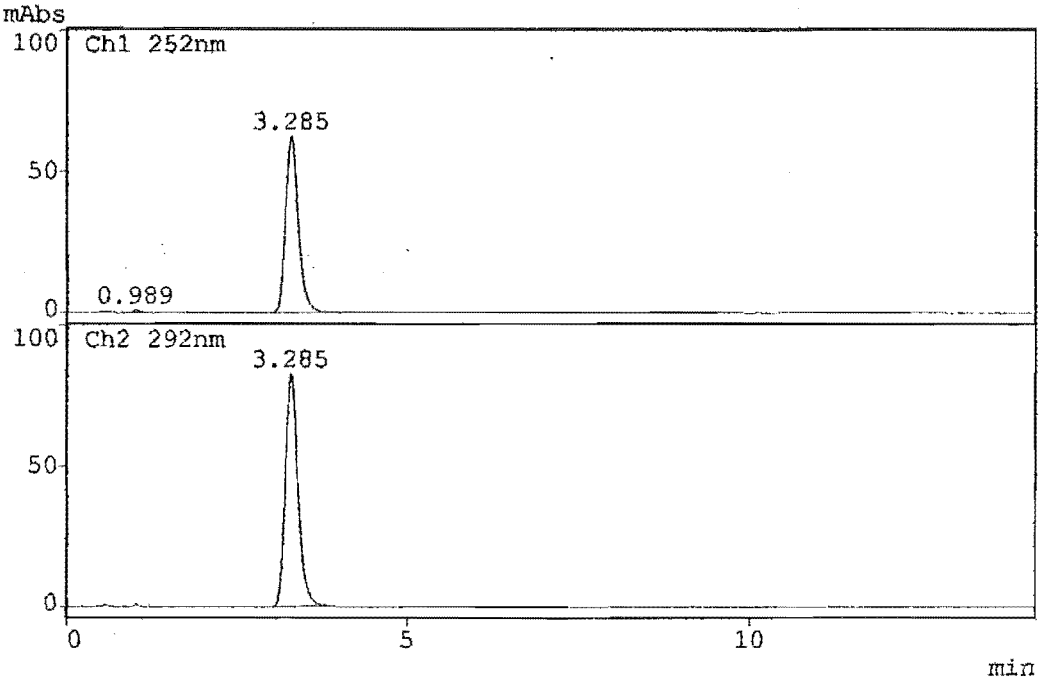


**Figure 3-33. HPLC chromatogram on a C18 column of 8-hydroxycoumarin.** Analysis performed with 60:40 (v/v) water-methanol mobile phase. Examined at 252 and 292 nm.

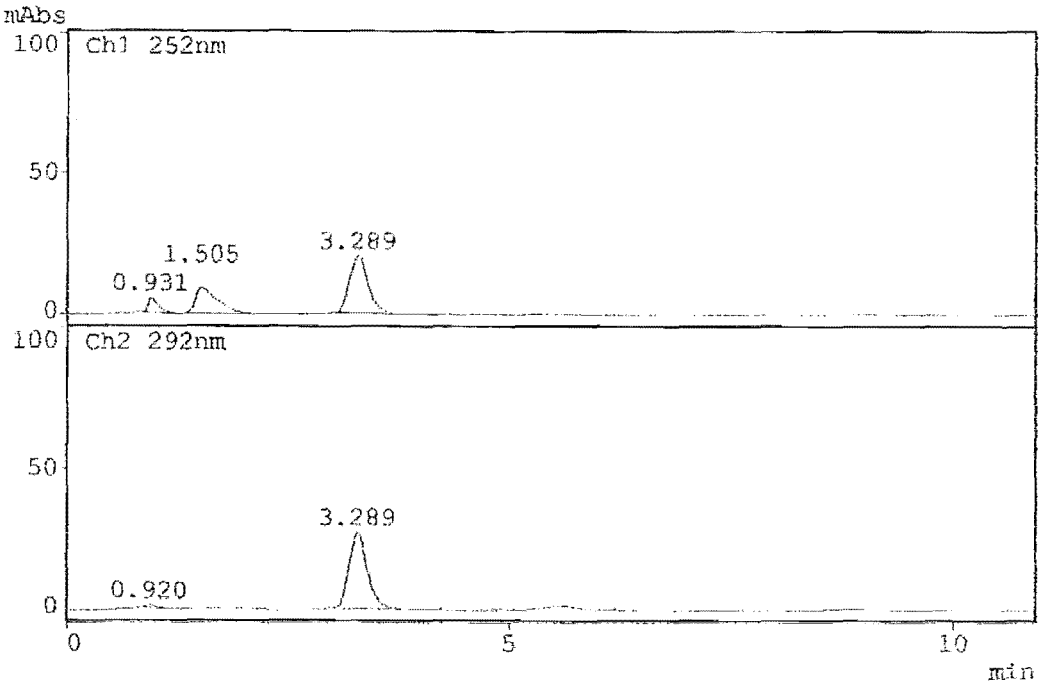


**Figure 3-34. HPLC chromatogram on a C18 column of ethyl acetate soluble material extracted from the culture medium of OT11 pRO1614.** Analysis performed with 60:40 (v/v) water-methanol mobile phase. Examined at 252 and 292 nm.

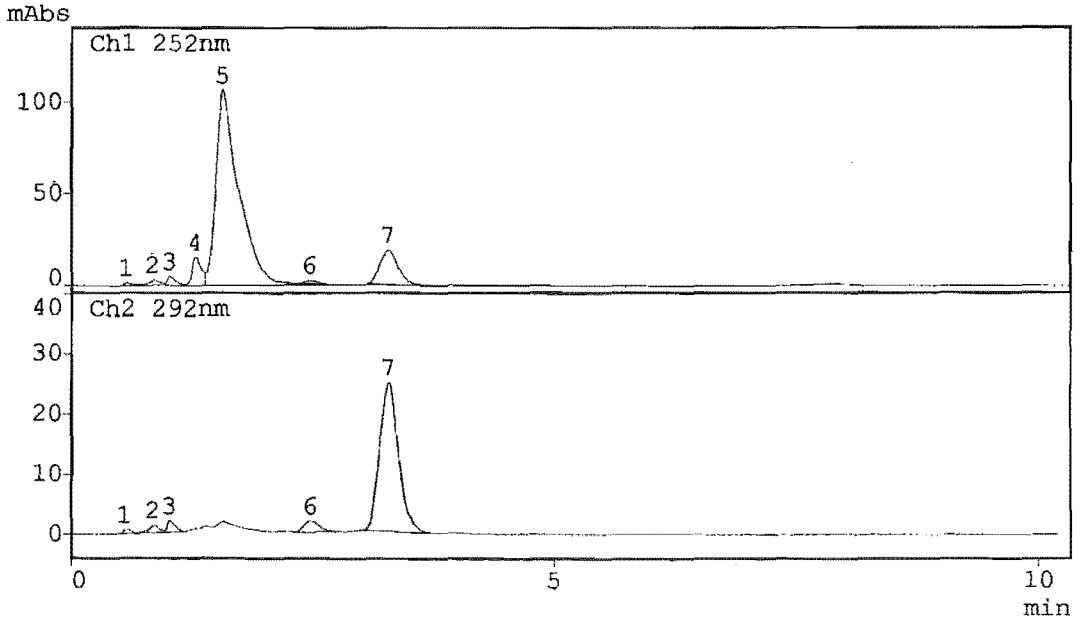


**Figure 3-35. HPLC chromatogram on a C18 column of ethyl acetate soluble material extracted from the culture medium of OT11 pNHQR4.** Analysis performed with 60:40 (v/v) water-methanol mobile phase. Examined at 252 and 292 nm. Retention times (min) of peaks are as follows: 1, 0.55; 2, 0.84; 3, 1.01; 4, 1.27; **5, 1.55**; 6, 2.47; **7, 3.29**.

The results of the HPLC analysis indicate that OT11 pRO1614 does not metabolise 8-hydroxycoumarin as the characteristic peaks in absorbance at 252 and 292 nm (Figure 3-34) correspond to the same retention times as is observed for 8-hydroxycoumarin (Figure 3-33). With OT11 pNHQR4 a new peak at 252 nm is observed with a shorter retention time of 1.55 min (Figure 3-35, peak 5). It is apparent that 8-hydroxycoumarin remains in the in the medium, however, given by the presence of peak 7. This was confirmed by analysing the UV spectrum of the eluted products represented by peak 5 and peak 7 (Figure 3-36).

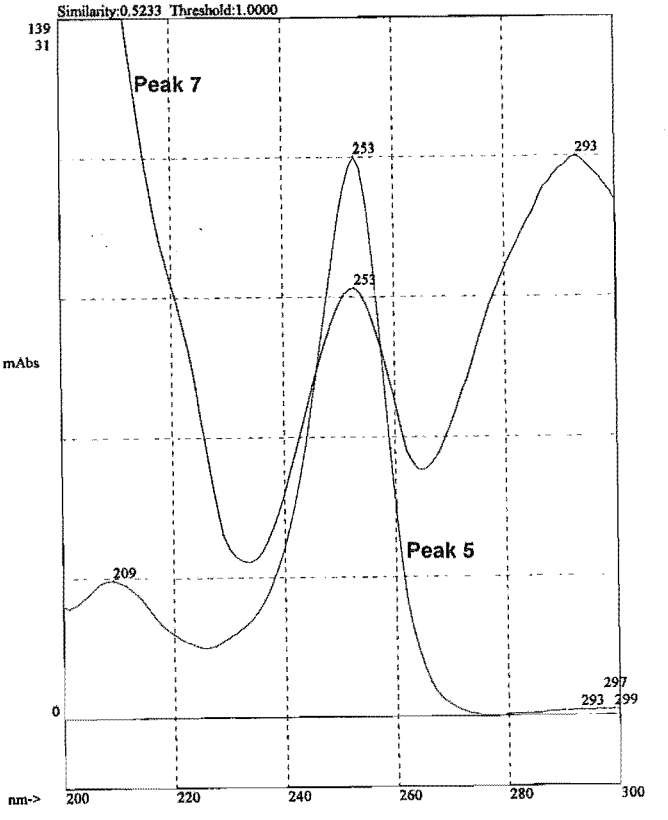


**Figure 3-36. Overlayed UV spectra of eluted compounds from HPLC analysis.** UV spectra of eluted peak 5 and peak 7 from HPLC chromatogram (Figure 3-35).

It can be concluded that when OT11 pNHQR4 is incubated in the presence of 8-hydroxycoumarin, a new compound accumulates in the culture medium, which is more polar than 8-hydroxycoumarin and contains a less extended UV chromophore at 292 nm. However, conversion to the new compound is not

complete as a small proportion (not detectable in the original UV spectrum (Figure 3-32)) of 8-hydroxycoumarin remains in the culture medium.

## 3.11 DNA SEQUENCE ANALYSIS

The full nucleotide sequence (Sequence 1, depicted as the 'positive' strand) derived from DNA sequence analysis of the *Eco*RI fragment from pNHQ83 and primer walking in pC1 and pNHQ84 of 3128 bp is shown in Appendix IV. Predicted (DNAMAN) restriction sites within this sequence appear in Appendix V. Analysis of Sequence 1 data using DNAMAN revealed 6 potential open reading frames (ORF) in both positive strand (Figure 3-37) and negative strand (Figure 3-38). Note positive and negative strands are shown separately for ease of interpretation (exact positions of potential ORFs appear in Appendix VI).

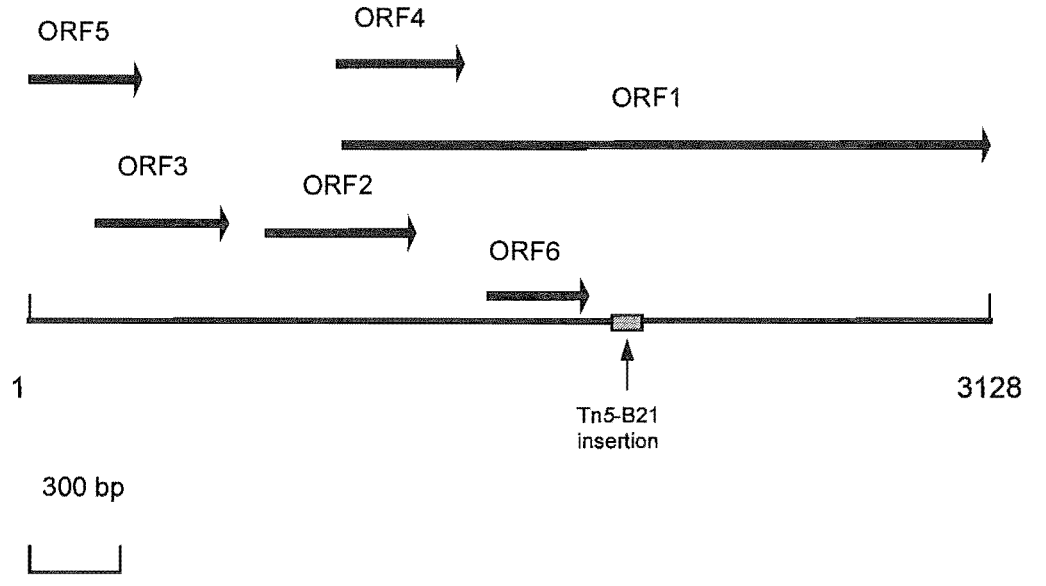


**Figure 3-37. Putative open reading frames in Sequence 1 (positive strand) identified by DNAMAN.** Depicted DNA strand reads 5'-3'. Grey box shows region of transposon insertion in equivalent QP100-8 sequence.

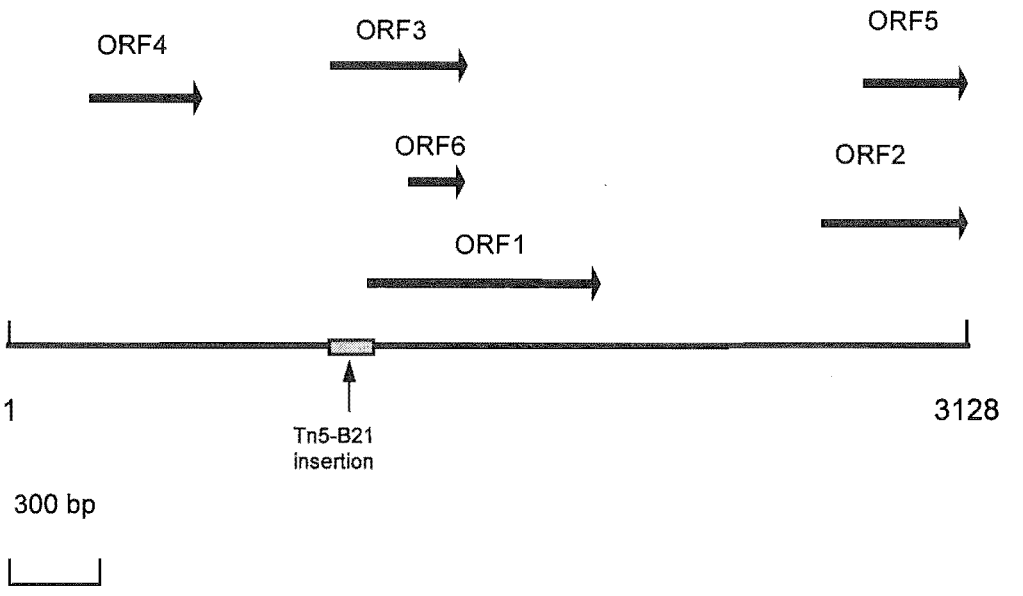


**Figure 3-38. Putative open reading frames in Sequence 1 (negative strand) identified by DNAMAN.** Depicted DNA strand reads 5'-3'. Grey box shows region of transposon insertion in equivalent QP100-8 sequence.

DNA sequence similarity searches between Sequence 1 and those stored at NCBI Blast server (Genebank database) were performed using the blastn and blastx algorithms (Altschul et al. 1990; Altschul et al. 1997). The FASTA algorithm (Pearson and Lipman 1988) was also used in this manner. No significant matches were found between Sequence 1 and those stored at the databases. Similarly, protein translations of the potential six reading frames in Sequence 1 did not significantly match any of those stored at these databases.

# 4. DISCUSSION

## 4.1 RESTRICTION BARRIER IN QP STRAINS

Experiments to introduce Tn*5* into QP100 via a mobilisable suicide vector from *E. coli* initially failed to produce $Km^R$ or $Sm^R$ resistant colonies at a level greater than the spontaneous resistance of QP100 to these markers. To investigate a possible reason for failure to transfer the transposon, several interspecific mating experiments with RP4 were performed with QP100 and QP101. It was found from these experiments that RP4 could successfully be transferred to QP100 strains from other *P. aeruginosa* strains but not from *E. coli*. It was also observed that QP101(RP4) was 400 times more efficient at donating RP4 than another *P. aeruginosa* to QP101. Similar results were also observed in transformation experiments using pRO1614 which had been isolated from an *E. coli*, *P. aeruginosa* (OT684) or QP100 background and then used to transform QP100 cells. Plasmid DNA isolated from *E. coli* did not transform QP100 cells. If the DNA was first used to transform OT684 then re-isolated from this new host, transformants of QP100 could be isolated from this DNA, though at a very low level ($4\times10^{-5}$ less frequently than in OT684). Finally, DNA isolated from QP100 would transform naive QP100 cells 500 times more efficiently than DNA isolated from OT684. These transformation experiments coupled with the RP4 conjugation studies may be interpreted to suggest that QP strains have a restriction barrier to DNA entry from *E. coli* and to a lesser extent other *P. aeruginosa*. An interpretation of the observation that DNA isolated from QP transforms QP more efficiently than the same DNA molecule isolated from OT684 (or *E. coli*) could be that QP strains destroy DNA recognised as foreign from non-QP strains. This would require that DNA synthesised in QP strains can be recognised by QP strains and therefore not be destroyed.

The successful introduction of Tn*5*-B21 into QP100 following growth of this recipient at 43 °C, contrasts with the inability to isolate QP100 mutants when this higher growth temperature was not applied to the recipients. A simple heat

shock at 50 °C following growth of QP100 at 37 °C did not provide successful introduction of Tn5-B21 into QP100 (data not shown). This result suggests that growth at 43 °C causes a greater phenotypic change than thermal inactivation of a nuclease. Holloway (1965) showed that by growing *P. aeruginosa* at 43 °C DNA for at least 5 generations, restriction was partially inactivated for up to 60 generations at 37 °C. As the return to the permissive phenotype was slow, it was concluded that simple inactivation of enzymes at 43 °C cannot explain the result. The observations with growth of QP at 43 °C are consistent with those of Holloway (1965).

## 4.2 MOLECULAR ANALYSIS OF QUINOLINE DEGRADATION IN QP

### 4.2.1 Plasmid association with quinoline degradation in QP

Plasmids have often been associated with microbial degradation of hydrocarbons and PAHs (Chakrabarty 1976) and some quinoline degraders (Brockman et al. 1989; Aislabie et al. 1990). Aislabie et al. (1990) showed that QP1 harboured 4 large plasmids, and plasmid curing experiments indicated that 2 of these plasmids (250 and 225 kb) were essential to quinoline degradation, a third plasmid (320 kb) contained further copies of sequences that coded for the first step of the quinoline degradation pathway. Attempts by (Horridge 1991) to repeat the isolation of these plasmids using a variety of techniques including the methods of Eckhardt (1978) and Casse et al. (1979) utilised by Aislabie et al. (1990) failed, while these methods proved successful for the isolation of large plasmids from other *P. aeruginosa* strains, however.

This work again addressed the matter of plasmid presence in QP strains. The association of quinoline degradation with plasmids, and particularly if multiple copies of any DNA sequences determining steps of the pathway would dictate the manner in which these genes could be studied. The plasmid preparation technique described by Kieser (1985) had been successfully applied to environmental isolates of various *Pseudomonas* species capable of hydrocarbon, PAH and heterocyclic compound degradation (Foght and

Westlake 1988). This method did not reveal the presence of plasmids expected of QP. It was successful for the isolation of two large plasmids (192 and >300 kb) from *Agrobacterium tumefaciens* and the isolation of RP4 from various *P. aeruginosa* strains including QP100. It was concluded from these results that the technique was suitable for the isolation of very large plasmids (i.e. shearing forces generated from this technique are not sufficient to destroy all the large DNA molecules) and that the method was suitable for isolating large plasmids from QP strains. Analysis of total genomic DNA of QP by CHEF electrophoresis isolated from cells that had previously heat treated) revealed intact chromosomal DNA but also failed to reveal the presence of large plasmids in this strain.

As plasmid preparations from QP had been negative for that characteristic, plasmid curing by the method described in Aislabie et al. (1990) was attempted. It was assumed that if plasmids could not be visualised directly, but given they were associated with quinoline degradation, their presence could be assayed indirectly, by curing of the plasmids and, therefore the $Qln^+$ phenotype. No $Qln^-$ cells could be isolated from these curing experiments indicating the phenotype was now stable to SDS treatment. Similarly no spontaneous $Qln^-$ QP strains were observed over the course of this study, which also points to the stability of this phenotype.

From the failure to both isolate the plasmids directly, and to remove the $Qln^+$ phenotype by plasmid curing, it is possible to conclude that presence of plasmids associated with quinoline degradation no longer exist in QP strains. The mechanism of loss of these plasmids is not known. Following the study (Aislabie et al. 1990), QP was stored at 4 °C in the presence of quinoline for 18 months (J Aislabie pers. comm.). It is possible that under constant quinoline selection the genes for quinoline metabolism may have been brought together, and perhaps are now even chromosomally located, in a similar manner suggested by Kellog et al. (1981) for the collection of genes encoding 2,4,5-trichlorophenoxy-acetic acid (2,4,5-T) degradation. In this study, a mixture of

microorganisms from various waste dump sites were grown in a chemostat with other bacteria harbouring various plasmids conferring aromatic compound degradation, these were cultivated for 8-10 months with increasing 2,4,5-T concentration over the time period. Eventually a consortium of bacteria were isolated that could utilise 2,4,5-T as a sole source of carbon. Further subculturing at higher 2,4,5-T concentrations led to a smaller consortium and finally a single colony was isolated capable of mineralising 2,4,5-T (Kilbane et al. 1982). This ability to mineralise 2,4,5-T, was assumed to be at least partially plasmid encoded, as the phenotype was not stable in the absence of selection.

#### *4.2.1.1 CHEF electrophoresis*

The technique of PFGE (pulsed-field gel electrophoresis) allows the separation and resolution of very large DNA molecules. The CHEF (contour-clamped homogeneous electric field) apparatus applies this technology with the added advantage that its hexagonal array of electrodes produce a uniform and homogeneous field across all lanes of the gel. Genomic DNA is liberated from bacteria while immobilised in agarose to minimise physical shearing forces on the large DNA molecules, intact chromosomes can be isolated in this fashion as well as large plasmid molecules. Alternatively, liberated DNA can be cleaved with rare recognition site restriction endonucleases and following PFGE, a macro-restriction map of the whole genome can be produced. The endonuclease, *Spe*I has been used in this fashion to produce a macro-restriction map of the 5.9 Mb *P. aeruginosa* PAO genome (Römling and Tümmler 1991). The *P. aeruginosa* PAO chromosome was found to produce 38 fragments with *Spe*I ranging in size from 517 kb to 5.2 kb, 25 of these fragments were 50 kb or greater.

The aim was to apply this technology to QP strains to perhaps reveal any plasmids that had not been visualised by the other techniques, compare the *Spe*I digest pattern of QP to that of other *P. aeruginosa* and to size the QP genome. These experiments were expected to reveal if the plasmids existed in the genome of QP, their total size of approximately 1 Mb would be detected

and this would presumably make a marked change the *Spe*I banding pattern relative to other *P. aeruginosa*. DNA sequences mutagenised with transposons could also be located to specific *Spe*I fragments by Southern analysis.

The technique described in Römling and Tümmler (1993) for isolation of total genomic DNA in *P. aeruginosa* failed to isolate large DNA molecules in QP. The result of this preparation seen in Figure 3-6 was typical for many preparations from QP, only DNA of <50 kb remained, while this technique was successful for other *P. aeruginosa*. This apparent self digestion of chromosomal DNA was also noted when performing standard genomic DNA preparations such as in the Owen and Borman (1987) technique, DNA had to be thoroughly extracted with phenol to prevent loss of the larger Mr DNA. It may be concluded from this that QP strains harbour an exonuclease that rapidly digests linear DNA molecules. Such activities have been observed in other *P. aeruginosa* strains (Potter and Loutit 1982). To test whether this phenomenon was heat-labile QP cells were cast in agarose and incubated at 75 °C for 10 minutes before loading onto an agarose gel or treatment with *Spe*I. The result in Figure 3-7 indicates this treatment had been partial successful, while some intact chromosomal DNA was visible on the gel, a quantity of partially digested "smeared" DNA was also apparent. This heat treatment of the cells may have partially inactivated the hypothesised exonuclease. Further manipulation of this incubation needs to be addressed to optimise the conditions for maximum large-DNA molecule isolation in QP strains. The use of heat-stable proteases to replace proteinase K and incubation in their presence at higher than 56 °C also might improve large molecular weight DNA recovery.

### 4.2.2 Transposon mutagenesis

A growth/non-growth plate assay, were quinoline was supplied as a sole source of carbon, nitrogen and energy, provided a rapid means to distinguish $Qln^+$ from $Qln^-$ cells. The putative $Qln^-$ colonies were then screened in a second assay in liquid QBHS, where the UV spectrum of the medium was periodically observed.

Any changes of the UV spectrum of the organism on test could be directly compared to uninoculated controls by simple overlaying of the spectra. This secondary assay was used to confirm the Qln$^-$ phenotype and determine its stability.

Once DNA restriction in QP100 was overcome, by growing this recipient at 43 °C DNA prior to mating with *E. coli* pSUP102 (Tn*5*-B21) donors, transposon mutagenesis was successful. $Tc^R/Gm^S$ transconjugants of QP100 were isolated, this indicated that successful integration of Tn*5*-B21 into the QP genome with the desired suicide of the vector plasmid (pSUP102, $Gm^R$). From 1100 of the $Tc^R$ colonies screened, 4 auxotrophic mutants and 5 mutants impaired in their ability to grow on quinoline were isolated. It is surprising to isolate almost as many auxotrophic mutants as quinoline growth impaired mutants. This may reflect hotspots for Tn*5* insertion in the "quinoline" genes or perhaps may be an artefact of mutagenesis, for example, multiplication of a quinoline deficient mutant.

Three of the 5 isolated quinoline growth-impaired mutants contain a transposon insertion in the same *Bam*HI and *Bgl*II fragments. Subject to the resolution of the Southern analysis of AGE of these enzyme digestions, the insertion appears to be in same sequence of DNA. Two possibilities for this observation are firstly, the 3 mutants might be a result of cellular replication of a single cell acquiring the transposon insertion and therefore are clones. Secondly, the possibility also exists that the 3 mutations are from independent mutation events and the inserted sequence contains an insertion 'hot spot' for Tn*5*. Insertion hot spots for Tn*5* insertions in *P. aeruginosa* and other fluorescent Pseudomonads have been reported in pyoverdine gene clusters (Cornelis et al. 1992) These two possibilities are impossible to distinguish from this experiment, further mutation experiments and DNA sequence analysis might provide data to conclude which is the more likely of the possibilities.

All the 5 quinoline growth-impaired QP100 mutants contain a single transposon insertion suggesting each observed phenotype is a result of a single transposon insertion event. Though it was not possible to rule out a second transposition event after initial insertion of the transposon in the QP100 genome, as the Tn*5* transposition mechanism is conservative, which would make detection of a second transposition event within a cell difficult by Southern analysis. Every effort was made to maintain Tc selection and single colony purity from the initial mutant isolation to minimise the possibility of secondary transposition.

In conclusion, this system of mutagenesis was shown to be successful in QP strains, though further work may give an insight into the stability and randomness of Tn*5* transposition in QP.

### 4.2.3 Phenotype of mutants

Of the 5 mutants impaired in their ability to degrade quinoline, QP100-1 and QP100-9 retained the ability to degrade quinoline at albeit at a reduced rate compared to wt QP100. These two mutants differ from one another in the position of transposon insertion and the extent to which their growth on quinoline is retarded. It is impossible to conclude whether the mutations caused by the transposon have affected genes of the quinoline degradation pathway directly or some other genes that indirectly affect the ability to grow on quinoline. The mutants grow as well as wild-type QP100 in complex media and M9 media indicating that general cellular metabolism is unaffected. With QP100-1, where quinoline degradation appears to be retarded a further 40 hr when compared to QP, an alternative explanation may be presented. In this mutant, the transposon may have inserted in a region of DNA which does not result in stable insertion for Tn*5*, such regions have been reported (Berg 1989). The observed delay of quinoline metabolism in QP100-1 may be based on the stochastic probability of transposon loss from this sequence of DNA. This

mutant may warrant further study, though the potential instable transposon insertions may hamper this.

QP100-6, QP100-7 and QP100-8 all failed to grow in QBHS media indicating they could no longer mineralise quinoline. When these mutants were grown in QBHS media supplemented with glucose the cells were able to partially metabolise quinoline, all 3 mutants accumulated a compound in the culture media with the same UV spectrum. A compound with the same UV spectrum was isolated from the culture media of QP100-8 cells, both the UV and the NMR spectra of this compound was that expected of 8-hydroxycoumarin.

Accumulation of 8-hydroxycoumarin by the QP100 mutants is evidence for its role as an intermediate of quinoline degradation by QP strains. This compound is an intermediate of the 'coumarin pathway' first described by Shukla (1986) for a *Pseudomonas* species. This pathway has also been described for other *Pseudomonas* species (Schwarz et al. 1989; Shukla 1989). The presence of 8-hydroxycoumarin as an intermediate in quinoline metabolism is evidence that QP strains degrade quinoline in a similar fashion to the aforementioned coumarin pathway. The transposon insertion presumably has affected the expression of an enzyme involved in the cleavage or lactonisation of the heterocyclic ring (Figure 1-7). This insertion may have affected a structural gene which directly codes for an enzyme to catalyse this process or perhaps a regulatory element which exerts an effect on this step of the pathway. Similarly, interruption of a multi-gene operon would affect the expression of genes downstream of the transposon insertion, it is impossible to determine the nature of the sequence containing the insertion from these results.

### 4.2.4 Complementation analysis

DNA complementation assays were performed with QP100-6, QP100-7 and QP100-8 each containing one of the plasmids pNHQR4, pNHQR2 and pNHQR8. Both pNHQR2 and pNHQR8 failed to complement the mutation in any of the $Qln^-$ mutants and return the phenotype to $Qln^+$. All 3 $Qln^-$ mutants

were able to grow on quinoline assay plate when transformed with pNHQR4. These complemented mutants grow approximately 50 % slower on quinoline than do wild-type QP. To restore the $Qln^+$ phenotype two possible explanations can be considered, either complementation of the mutant sequence is in trans and the wild-type gene product is supplied from pNHQR4 directly, or $Qln^+$ is restored by recombinational repair of the mutant sequence resulting in the excision of the transposon. Recombinational repair of the mutant sequence may be possible, as the homologue of the *E. coli recA* gene has been found in *P. aeruginosa* and this gene mediates many of the functions in *P. aeruginosa* as observed in *E. coli*, including homologous recombination (Ohman et al. 1985). If some form of homologous repair is involved with complementation by pNHQR4, it might also be expected that pNHQR2 should complement the mutants also. It would perhaps, be at a lower frequency, as less homologous DNA is supplied by this plasmid (4.5 kb vs 7.5 kb with pNHQR4). As pNHQR2 does not complement the mutants, then the level of homologous recombination with this plasmid is too low to detect. It might also be expected that complemented mutants would grow at the same rate on quinoline as the wild-type, if recombinational repair where responsible for the observed complementation. Complementation in trans might lead to a slower growing phenotype as the genes expressed in pNHQR4 are now longer in the 'context' of their position in QP, gene regulation, for example, could be affected. It is possible to tentatively conclude that pNHQR4 complements the $Qln^-$ mutants in trans, but further experiments to prove this and rule out recombinational repair would have to be performed to determine this with certainty.

### 4.2.5 Expression of DNA encoded by pNHQR4 in OT11

From the complementation experiments it was impossible to determine whether the recombinant plasmid pNHQR4 encoded a structural gene product. If this plasmid encoded an enzyme for the quinoline degradation pathway it would presumably act upon 8-hydroxycoumarin given by its accumulation in the culture media as a result of the transposon insertion. To test this hypothesis *P.*

*aeruginosa* OT11 pNHQR4 was grown in the presence of 8-hydroxycoumarin. Following this incubation, the UV spectrum of the ethyl acetate soluble fraction of the culture media was found to be modified from that of 8-hydroxycoumarin. This fraction contained a small number of new components as judged by reversed phase HPLC. In particular, the fraction with the most intense absorbance at 252 nm was a new compound, having a shorter retention time than 8-hydroxycoumarin. This material had a diminished absorbance at 292 nm, relative to 8-hydroxycoumarin, which is consistent with it possessing a less extended UV chromophore. This is expected for reduction of the oxygen containing heterocyclic ring structure. Furthermore, a new peak was observed in the IR spectrum of this material at 1693 $cm^{-1}$ (data not shown). This is in accordance with the reported IR data of 2,3-dihydroxyphenylpropionic acid. These results are consistent with a new compound being present in the culture media with the characteristics of 2,3-dihydroxyphenylpropionic acid, the next isolated intermediate in the quinoline degradation pathway. Unfortunately insufficient pure material was extracted from the culture media to generate reliable NMR and mass spectra data to identify the new compound with absolute certainty. There is therefore, direct evidence that the pNHQR4 plasmid encodes a protein that can act upon 8-hydroxycoumarin.

This experiment lends further weight to the hypothesis that pNHQR4 complements the Qln$^-$ mutants in trans as the plasmid encodes functional enzyme(s). It presently cannot be determined however, if the interrupted gene encodes this enzyme.

If 2,3-dihydroxyphenylpropionic acid is accumulating in the culture media as a result of enzymic modification of 8-hydroxycoumarin, then at least two distinct reactions must be occurring: reduction (hydrogenation) of the heterocyclic ring and oxidation of a ketone to corresponding carboxylic acid. Shukla (1989) describes this as a two reaction step, perhaps involving enzyme bound open ring structures (Figure 1-7). If two enzymes are involved, a reductase and an oxidase (hydroxylase), then presumably the gene coding for reductase has

been interrupted by the transposon as 8-hydroxycoumarin and not the lactone (reduced product) is detected in the culture media of QP100-8. However pNHQR4 must confer the oxidative function as well, as the lactone is not the detected new compound in OT11 pNHQR4 media. It should be possible to tease these two functions apart by subcloning portions of pNHQR4 and finding the minimum complementing fragment in QP100-8 then assaying the new plasmid in OT11 provided with 8-hydroxycoumarin. Similarly, saturation mutagenesis in pNHQR4 could be assayed in an identical manner. The results of these experiments might indicate if the conversion of 8-hydroxycoumarin to 2,3-dihydroxyphenylpropionic acid is mediated by a single enzyme or involve several. Minicell analysis and SDS-PAGE analysis of the 7.5 kb fragment contained in pNHQR4 would reveal data on the size and quantity of proteins encoded by this fragment. This data would also assist with identifying appropriately sized open reading frames in the DNA sequence data.

It can be concluded from this experiment that the pNHQR4 plasmid encodes a at least one enzyme that can act upon 8-hydroxycoumarin and convert it to a new product, and this protein is expressible and active outside of QP strains. This hypothesised enzyme(s) must have both oxidative and reductive capabilities as a double bond in heterocyclic ring is reduced and oxidation of a ketone to the carboxylic acid occurs.

### 4.2.6 DNA sequence analysis of cloned QP DNA

Approximately 3.2 kb of DNA sequence was generated (Sequence 1). This DNA did not significantly match any of those currently described and stored at Genbank.

Potential ORFs were identified in both the positive and negative strands of Sequence 1. In the positive strand (Figure 3-37), a potential large incomplete (no stop codon) ORF (ORF1) was identified, which in QP100-8 would be interrupted by the transposon. ORF1 shows promise as a potential candidate

encoding an enzyme, as this hypothesised protein would be at least 700 aa long. The other 5 potential ORFs in the positive strand would not be affected by the transposon insertion, so are presumably not involved directly with the accumulation of 8-hydroxycoumarin in QP100-8.

Of the potential ORFs identified in the negative strand, only ORF3 and ORF1 would be directly interrupted by the transposon insertion. However ORF3 would be provided complete by pNHQR2 and since this plasmid does not complement the mutants, it seems unlikely to be the structural gene (or a component). ORF4 would not be affected by the transposon insertion due to its relative upstream position. ORF1 begins at close to the point of transposon insertion and continues beyond the *Bam*HI site that delineates the fragment in pNHQR2, this might then code for a structural gene. ORF5 and ORF2 are both incomplete (no stop codon) and their expression would only be affected by the transposon insertion if it were regulated in an operon, as they lie at least 1.5 kb upstream from the site of the transposon insertion.

It should be noted that the sequence data generated does contain some unknown nucleotides and was only partially generated from both DNA strands. This data, therefore, may have shortcomings and base substitutions may lead to different ORF interpretations. Further generation of quality sequence data and codon use examination would assist in more accurate ORF predictions.

Interestingly, the nucleotide composition of Sequence 1 is approximately 62% G-C, this is lower than the average 68% G-C of *P. aeruginosa*. This may reflect the (historical) plasmid borne nature of quinoline degradation in this organism. *P. putida* capable of quinoline degradation are often isolated (Table 1-1), this organism has a G-C percentage of 62%. Both the *qor* and *oxo* genes, encoding the first and second enzymes in quinoline degradation, have been sequenced (Bläse et al. 1996 & Rosche et al. 1997) and both revealed a G-C percentage of 62. It is a tantalising, if not fanciful, possibility that the genes conferring quinoline degradation have originated in *P. putida* and have been

transferred horizontally to other organisms either in situ or during the isolation/enrichment of *P. aeruginosa* QP in vitro. The possibility remains, of course, that the G-C percentage observed here is coincidentally similar to *P. putida* and simply represents a lower than average G-C sequence in *P. aeruginosa*. Codon use analyses could be performed to provide further evidence for this hypothesis.

The DNA sequence generated here might be applied in gene-probe technology for the detection of quinoline ring cleavage genes (enzymes) in situ. It has been noted (Pereira et al. 1987) that quinoline degradation metabolites such as 2(1H)quinolinone occur in groundwater, which are more mobile than the parent quinoline. Ring cleavage of these compounds is important for their rate of degradation. The ability to detect whether the biological capability exists in a polluted environment for a compound to be degraded is important to determine the course of action for clean up of the site. Gene-probes in combination with polymerase chain reaction (PCR) technology can be used to detect whether this biological potential exists at a site (Steffan and Atlas 1991) without the need or ability to culture microorganisms.

It is desirable to sequence the entire 7.5 kb insertion in pNHQR4 to complete this data. As it has been concluded that this insertion encodes (an) enzyme(s) which act upon 8-hydroxycoumarin, this would only be the third described gene structure of a DNA sequence encoding an enzyme of the quinoline pathway. Further sequence data may assist with the enzymology of this step in the pathway, features such as nucleotide binding domains found in other redox enzymes might be identified, for example.

## 4.3 CONCLUSIONS

Molecular analysis of *P. aeruginosa* QP has been successful in revealing the nature of quinoline metabolism in this organism. It appears that the genes for quinoline metabolism are no longer plasmid encoded, though DNA sequence

analysis revealed evidence of non *P. aeruginosa* origin of this DNA. Fine-tuning of the whole genome isolation of QP strains to allow successful CHEF electrophoresis would enable a *Spe*I restriction map of QP to be produced. The location of genes encoding quinoline metabolism on this map could be ascertained and compared to established *P. aeruginosa* physical and genetic maps.

Analysis of transposon mutants revealed the accumulation of 8-hydroxycoumarin in the growth media, as a by-product of quinoline metabolism, indicated this organism utilises the coumarin pathway of quinoline metabolism, where the heterocyclic ring of quinoline is first cleaved. Further transposon mutagenesis experiments could reveal more genes in the quinoline degradation pathway. Only the genes encoding quinoline oxidoreductase and 2-oxo-1,2-dihydroquinoline 8-monoxygenase have been described thus far. In addition, these experiments might reveal further unidentified intermediates in quinoline degradation pathway. The mechanism of nitrogen removal form the heterocyclic ring and the fate of to 2,3-dihydroxyphenylpropionic acid are still unknown (Schwarz and Lingens 1994).

Expression of an enzyme encoded by a fragment of DNA which complemented the Qln$^-$ mutants, in OT11 (a non-quinoline degrading *P. aeruginosa*) enabled this strain to mediate the conversion of 8-hydroxycoumarin to 2,3-dihydroxyphenylpropionic acid. Minicell and SDS-PAGE analysis would reveal the size and number of proteins expressed from this fragment. High level expression of proteins encoded by this fragment would allow for enzymology studies. It seems likely this fragment encodes an enzyme which catalyses the opening of a heterocyclic ring structure, the nature of this reaction is not currently known (Schwarz and Lingens 1994) and therefore further study of the enzyme(s) encoded by this plasmid is warranted.

The 3.2 kb of DNA sequence generated from the clones in this study was found to be previously undescribed in sequence databases. Several potential ORFs

were identified in this data. Completion of sequencing of this fragment would enable the ORFs to be identified. A complete DNA sequence of the complementing clone is desirable as this plasmid encodes a function. The genes encoding quinoline oxidoreductase and 2-oxo-1,2-dihydroquinoline 8-monoxygenase are the only genes encoding quinoline degradation sequenced to date. It is not known where in the cell quinoline metabolism takes place. All the studies describing pathways of quinoline metabolism isolated transient intermediates from the culture media. This suggests the possibility of the enzymes being located in the cell periplasm. DNA sequence analysis may reveal transmembrane motifs for these enzymes and provide a clue as to their location in the cell, for example.

It should be possible to clone the >23 kb *Hin*dIII observed in Figure 3-19 into a cosmid vector and extend this with uninterrupted DNA from pNHQ8, in a similar fashion to the construction of pC1 and pNHQR4. This would provide nearly 30 kb of DNA from QP100. Bläse et al. (1996) found a cosmid containing a 30 kb fragment from *P. putida* 86 that could confer quinoline degradation in other *P. putida*. This assembled large insert could be analysed for further genes encoding quinoline metabolism. However, it is not known whether the quinoline degradation genes in QP are in close arrangement.

The results and generated materials presented here provide a base for further molecular analysis of the quinoline degradation pathway. An increased understanding of this process can be applied to the biodegradation of other heterocyclic compounds both in situ and in vitro.

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# APPENDIX I. Media

## Bushnell-Haas Medium

| g/l | |
|---|---|
| 1.0 | $K_2HPO_4$ |
| 1.0 | $KH_2PO_4$ |
| 0.20 | $MgSO_4.7H_2O$ |
| 0.05 | $FeCl_3$ |
| 0.02 | $CaCl_2$ |

pH adjusted to 7.0, autoclaved

## Trace element stock solution (100x) from (Schwarz et al. 1988)

| g/l | |
|---|---|
| 0.50 | $H_3BO_3$ |
| 0.04 | $CuSO_4.5H_2O$ |
| 0.20 | $FeCl_3.6H_2O$ |
| 0.40 | $MnSO_4.4H_2O$ |
| 0.20 | $(NH_4)_6Mo_7O_{24}.4H_2O$ (Ammonium Molybdate) |
| 0.40 | $ZnS0_4$ |
| 0.10 | KI |

autoclaved

## QBHS

| | |
|---|---|
| 99 ml | Bushnell-Haas |
| 1 ml | Trace element solution |
| 30 µl | Quinoline |

## Medium B (Schwarz et al. 1988)

| g/l | |
|---|---|
| 0.79 | $K_2HPO_4$ |
| 0.20 | $MgSO_4.7H_2O$ |
| 0.02 | $FeSO_4.7H_2O$ |

1.00 NaCl

autoclaved, 10 ml/l trace element solution added before use

**Medium C (Shukla 1986)**

**g/990 ml**

4.26 g $Na_2HPO_4$

2.65 g $KH_2PO_4$

autoclave, then add 10 ml of following stock solution

**g/100 ml**

2.0 $MgSO_4.7H_2O$

0.26 $MnSO_4.4H_2O$

0.50 $FeSO_4.7H_2O$

0.27 $CaCl_2$

0.01 Sodium molybdate

adjusted to pH 3.0 with $H_2SO_4$, then filter sterilised

**Luria Betani medium** (LB)

1.0% w/v Bactotryptone

0.5% w/v Yeast extract

0.5% w/v NaCl

In $dH_2O$, autoclaved

**SOC medium**

2.0% w/v Bactotryptone

0.5% w/v Yeast extract

10 mM NaCl

2.5 mM KCl

10 mM $MgCl_2$

10 mM $MgSO_4$

20 mM Glucose

In $dH_2O$, autoclaved

**M9 minimal medium**

5×M9 salts

| | |
|---|---|
| 64.0 g | $Na_2HPO_4.7H_2O$ |
| 15.0 g | $KH_2PO_4$ |
| 2.5 g | NaCl |
| 5.0 g | $NH_4Cl$ |

In 1 l $dH_2O$, autoclaved

**M9 medium**

| | |
|---|---|
| 200 ml | 5×M9 salts |
| 2 ml | 1M $MgSO_4$ (autoclaved) |
| 20 ml | 20% w/v glucose (autoclaved) |
| 1 ml | 1M $CaCl_2$ (autoclaved) |

to 1 l in $dH_2O$.

# APPENDIX II. Buffers and solutions

## *Common Buffers and solutions*

**TE**

| | |
|---|---|
| 10 mM | Tris-HCl (pH 8.0) |
| 1 mM | EDTA (pH 8.0) |

In $dH_2O$, autoclaved

**TAE**

| | |
|---|---|
| 50 mM | Trizma base |
| 1 mM | EDTA |
| 0.11% w/v | Glacial acetic acid |

In $dH_2O$

**TBE**

| | |
|---|---|
| 50 mM | Trizma base |
| 50 mM | Boric acid |
| 1 mM | EDTA |

In $dH_2O$

**DNA loading buffer** (for agarose gel electrophoresis)

| | |
|---|---|
| 80% v/v | Glycerol |
| 20 mM | EDTA (pH 8.0) |
| 0.025% w/v | Bromophenol blue |

in $dH_2O$

**20xSSC**

| | |
|---|---|
| 175.3g | NaCl |
| 88.2g | Sodium citrate |

in 1L $dH_2O$, pH brought to 7.0, autoclaved

**Phenol (pH 8.0)**

Crystalline phenol dissolved to saturation in 0.1 M tris-HCl pH 8.0
after pH is brought to 8.0 with 0.1 M tris pH 8.0

**Chloroform**

| | |
|---|---|
| 96% v/v | Chloroform |
| 4% v/v | Isoamyl alcohol |

**Phenol:Chloroform**

| | |
|---|---|
| 50% v/v | Phenol (pH 8.0) |
| 48% v/v | Chloroform |
| 4% v/v | Isoamyl alcohol |

***Specific Buffers and reagents for various protocols***

**Alkaline lysis plasmid mini-preparation and large scale plasmid extraction**

Solution 1

| | |
|---|---|
| 50 mM | Glucose |
| 25 mM | Tris-HCl (pH 8.0) |
| 10 mM | EDTA (pH 8.0) |

in $dH_2O$, autoclaved

Solution 2

| | |
|---|---|
| 1.0% w/v | SDS |
| 0.2 M | NaOH |

in $dH_2O$

Solution 3

| | |
|---|---|
| 60 ml | 5 M Potassium acetate |
| 11.5 ml | Glacial acetic acid |
| 28.5 ml | $dH_2O$ |

autoclaved

**Lysis by Boiling Plasmid Preparation**

Lysozyme solution

| | |
|---|---|
| 0.25 M | Tris-HCl (pH 8.0) |
| 10 mg/ml | Lysozyme |
| in $dH_2O$ | |

STET

| | |
|---|---|
| 0.1 M | NaCl |
| 10 mM | Tris-HCl (pH 8.0) |
| 1 mM | EDTA (pH 8.0) |
| 5% v/v | Triton X-100 |
| in $dH_2O$, autoclaved | |

**Large plasmid minipreparation**

Lysis solution

| | |
|---|---|
| 25 mM | Tris-HCl (pH 8.0) |
| 25 mM | EDTA (pH 8.0) |
| 0.3 M | Sucrose |
| in $dH_2O$, autoclaved, then add 2 mg/ml lysozyme | |

Alkaline/SDS

| | |
|---|---|
| 0.3 M | NaOH |
| 2.0% w/v | SDS |
| in $dH_2O$ | |

Acid-phenol

| | |
|---|---|
| 5 g | Crystalline phenol |
| 5 ml | Chloroform |
| 1 ml | $dH_2O$ |
| 8 mg | 8-hydroxyquinoline |

**Total genomic DNA preparation**

SE buffer

| | |
|---|---|
| 150 mM | NaCl |
| 100 mM | EDTA pH 8.0 |
| in $dH_2O$, autoclaved | |

SET buffer

| | |
|---|---|
| 150 mM | NaCl |
| 15 mM | EDTA |
| 60 mM | Tris-HCl |

in $dH_2O$, autoclaved

| | |
|---|---|
| 50 μg/ml | RNase A |

**CHEF electrophoresis reagents**

SE

| | |
|---|---|
| 75 mM | NaCl |
| 25 mM | EDTA (pH 8.0) |

in $dH_2O$, pH 7.4, autoclaved

Lysis buffer

| | |
|---|---|
| 0.5 M | EDTA |
| 1% w/v | *N*-lauroylsarcosine |

in $dH_2O$, pH 9.6, autoclaved

| | |
|---|---|
| 0.5 mg/ml | Proteinase K (Boehringer) added before use |

**Vacuum transfer reagents**

Depurination solution

| | |
|---|---|
| 0.25 M | HCl |

Denaturation solution

| | |
|---|---|
| 1.5 M | NaCl |
| 0.5 M | NaOH |

Neutralisation solution

| | |
|---|---|
| 1.0 M | Trizma base |
| 2.0 M | NaCl |

adjust to pH 5.0 with HCl

**Hybridisation solutions**

50x Denhardt's reagent

| | |
|---|---|
| 5g | Ficoll |
| 5g | Polyvinylpyrrolidone |

| | |
|---|---|
| 5g | Bovine serum albumin |
| 500 ml | $dH_2O$ |
| autoclaved | |

Prehybridisation solution

| | |
|---|---|
| 6x | SSC |
| 0.5% w/v | SDS |
| 100 μg/ml | Denatured fragmented herring sperm DNA |
| 5x | Denhardt's buffer (for low abdunce target sequences) |
| or | |
| 0.05x | BLOTTO (for plasmid clone targets) |

Hybidisation solution

| | |
|---|---|
| 6x | SSC |
| 0.5% w/v | SDS |
| 100 μg/ml | Denatured fragmented herring sperm DNA |

**Colony hybridisation reagents**

Denaturing solution

| | |
|---|---|
| 0.5 M | NaOH |
| 1.5 M | NaCl |

Neutralising solution

| | |
|---|---|
| 1.5 M | NaCl |
| 0.5 M | Tris-HCl (pH 7.4) |

BLOTTO

| | |
|---|---|
| 5% w/v | non-fat dried milk powder |
| in $dH_2O$ | |

Prewashing solution

| | |
|---|---|
| 5x | SSC |
| 0.5% w/v | SDS |
| 1 mM | EDTA (pH 8.0) |
| in $dH_2O$ | |

Pre/hybridisation solution

| | |
|---|---|
| 6x | SSC |

0.05x BLOTTO

in $dH_2O$

## Acrylamide Sequencing Gel

To make a 5 % acrylamide gel:

5 ml 10×TBE

5.95 ml 40% Acrylamide solution (Bio-Rad)

6.25 ml 2 % Bis-acrylamide solution (Bio-Rad)

21 g Urea

$dH_2O$ to 50 ml

The solution was filtered through Whatman® no.114 filter paper and degassed for 10 min. To polymerise the acrylamide 30 µl each of TEMED and 25% w/v ammonium persulfate were added prior to addition of the solution to gel mould.

# APPENDIX III. pNHQ8 fragment sizes

Specific restriction endonuclease fragment sizes from pNHQ8 (Figure Appendix III-1) appear Table Appendix III-1. Sizes were calculated using DNAfrag from measurements of restriction fragments from AGE of enzyme digests of pNHQ8.

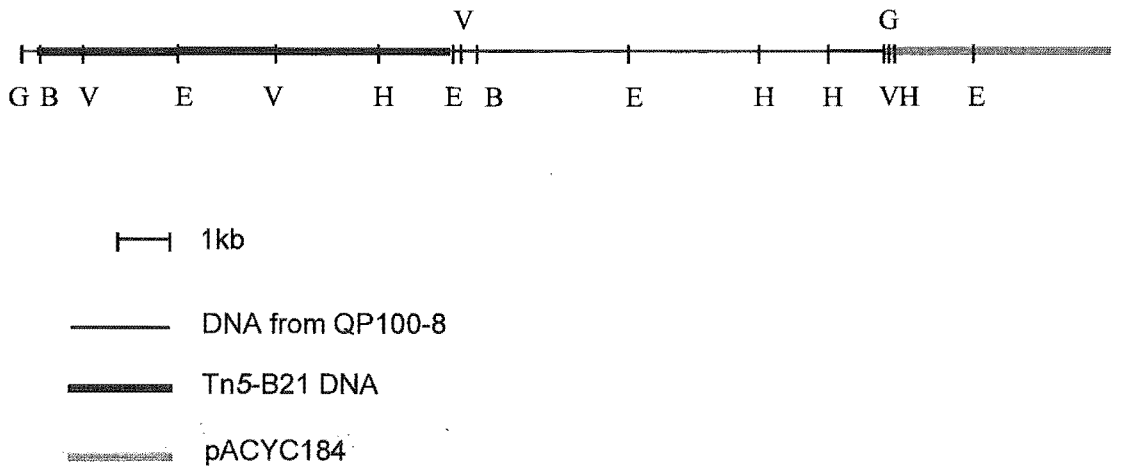


**Figure Appendix III-1. Linear restriction map of pNHQ84.** Restriction enzymes: B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII and V, *Eco*RV.

**Table Appendix III-1. Restriction enzyme digests of pNHQ84 fragment sizes.** Fragments whose size are known by sequence analysis appear in italics, those containing pACYC184 DNA are shown in bold.

| Enzyme treatment | Fragment Size (kb) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ***Bam*HI** | **12.26** | 8.71 | | | | | | |
| ***Hin*dIII** | **10.72** | 6.50 | **1.95** | 1.71 | | | | |
| ***Eco*RI** | **7.63** | **5.63** | 5.16 | 3.05 | | | | |
| ***Eco*RV** | 8.96 | **5.34** | 3.73 | 3.27 | | | | |
| ***Bam*HI/*Hin*dIII** | 6.99 | 4.97 | **4.05** | **1.95** | 1.74 | 1.2 | | |
| ***Eco*RI/*Hin*dIII** | **5.75** | 3.87 | 3.10 | 2.29 | **1.95** | 1.74 | **1.50** | 1.26 |
| ***Eco*RI/*Eco*RV** | **6.01** | **3.67** | 3.24 | 2.98 | 1.92 | 1.90 | ***1.679*** | *0.100* |
| ***Bam*HI/*Eco*RV** | 8.96 | **4.33** | 3.87 | 3.36 | 1.1 | *0.364* | | |
| ***Bam*HI/*Eco*RI** | **7.82** | 5.28 | 3.03 | **2.63** | 2.56 | *0.464* | | |
| ***Hin*dIII/*Eco*RV** | 5.45 | **5.34** | 3.80 | 1.95 | 1.80 | 1.75 | 1.46 | *0.156* |

# APPENDIX IV. DNA SEQUENCE DATA

DNA sequence analysis was performed on the inserts in pC1, pNHQ83 and pNHQ84, a diagrammatic summary of these reactions appears in Figure Appendix IV-1.

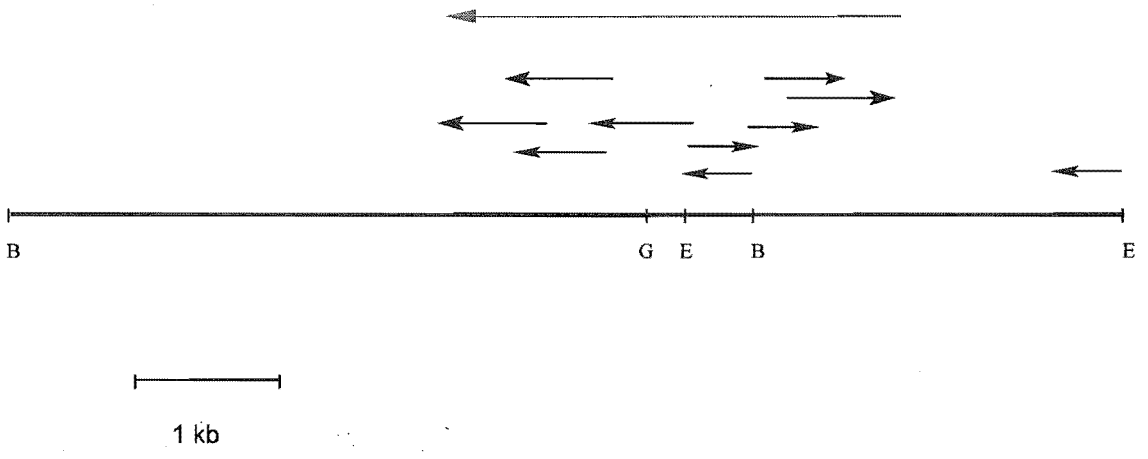


**Figure Appendix IV-1. Restriction map of the 7.5kb insert of QP100 DNA in pNHQ4R.** Black arrows show direction of sequence reactions performed on equivalent sequence in pC1, pNHQ83 and pNHQ84, but is shown on this one insert for clarity. Grey arrow shows direction of data (Sequence 1) presented below Figure Appendix **IV-2**. Restriction enzymes: B, *Bam*HI; E, *Eco*RI and G, *Bgl*II.

Sequence 1 (positive strand): 3128 bp;

Composition 528 A; 896 C; 1037 G; 596 T; 71 OTHER

Percentage: 16% A; 28% C; 33% G; 19% T; 2%OTHER

```
1     GCNTNANAAN ATCNGGGACN CCGTTTGCNG NGAGNANAAA GGGCCCGNGG GGTTTNCGCN
61    GGTTNGGTTG CCCGAATAAG GGNTCCCGGN NGACCNCTGG GCGTTTGGNG GAAGGGCNAC
121   CAGGTTNCCC NACCGAAGGA ANGGGTTNGN AGGNCGNANG CCATTTGNNG NATTGAATCC
181   CNAGNGAAGG CGGCANCGNC ANGAATAANT GGANTGGNNC AATGGGTGGG AAAAAGAAAG
241   CCCGGGTTTN GGGTTNCCCC AAGGGCCCAA GCGGTANGCC CAAGNNGGNC CCGGGGAATG
301   NCCAANGGCG GGNTNTNAAG CCGGCATTCA CCNGGAATGA TCGGGGCAGN ATGCACGAAA
361   TGAGTCGGGC AGATCGGGGT CCGNCNCCGG GCAGCAAGGC CGCCANCAGC GAANCAGGCT
421   TGTCCCNGCA AGCCTGGTCA NGTATCACCG ACTCGGGCGG AAGACGCATC CCTGAGTGCN
481   GGNCAAGTGC GCCAGCGCAG CCAGGACCTG CAGGTCCTGG CAGGACGGCT CAATACCTTG
541   GTGGGCCGTT TCAGGCTGTG AGCGNCTGCG CCACTGTTCC CACTCATCGC CGCACGGCCC
601   CTTCCAGGGG CGTTCTGTTA TCCGCGCGGT GCCGTGGGTG GACATGAATA TCTGCGCCCG
661   GGGGACAAGC CGTGCAGTGG CGGGATAGTT CGCCTGACGG GGCGTACCTA AAGTTGAGTT
```

```
721   GCCTGCCTTG TCAGGCGATC CACCACAAGA AATCGACGCA GAAACCGTGG ACTCGCCCTA
781   TGTCAAATCT CAGCAACATC GATCAATCCA TATCCCTGCG GATCTCGCGA CCGAGTGCCG
841   CGCAGCGCGC CAGCAAGGCG CTGCTGGCGG TGCTGCCCTG GGCGATCATC GCCGGTCTGC
901   TCTGGGCCGG GCTGTTCATC AAGCCGCAGC CGGTCGGCGG GACCGTCACC CCTCCGGCGC
961   TGGAACGCCG GGATCATTTC TACGGTCTGG CCCTCACGCC CGCTGGCGAC GTATGGGCCA
1021  GCGGCTCCAA TGGCAAGATC CTGGCGATCG CCGCCGATGG TGCCATCAAG CGGCTGGCGA
1081  CGCCCACCGA GCTTACGCTG CAGGACATCG CCATCTGGGA TGCCAGCCAT GCCGTGGCGG
1141  TCGGCAACGA TGGCGTGGTC CTGTACAGCC AGGATGCGGG TGGGAACTGG AAGGCAGCTA
1201  CCGGAGTGCC GCCTCGGACG TGGCGAACAA GCTCAATCGG GTGCGAATCG CCCCGGGTGG
1261  CGTAGCCATC GCCACCGGAG AGATGGGCGC ACTGCTGATC AGCCCGATTA CGGCCGGAGN
1321  NGGCAGCGCN TGCGCGAGGA GGAGGATGTG GCCTGGAACG ATGTGGCCAT TCTCGAGGAC
1381  GGCCGCCTGG TGGTGGTGGG NGAGTTCGGT CGGATCCTGA TCGGCGATCT CCAGGGGCGG
1441  CAATGGGAAG AGCCGGAACC GCCGGTTCCT GGTTCGCTGA TGTCGGTGCA GTTCCGCGAT
1501  GCGCAGAACG GTGTTGCCGT CGGCCTGGAA GGGGCCTTGC TGGTTACCCG TGATGGTGGC
1561  CACAGCTGGG AAAGCGTCGA CCTGGGGATG GCCGATCACC TGTTCGACGT CCTCTGGATT
1621  GCGCATCAGG GACGCTGGTT CATTAGCGGT GCAGTCGGGC GCTGGGCGTT CGGTGGGGGC
1681  GAGGGCTGGA AGACCGGCGT TCTGGATGAG CGCAGCCGTG CCTGGCACAC CCGCGCCCTG
1741  GTCGTCGGGT CTGACATCTG GCTGGCCGGC GCCGATATCG GCCGCTGGAA CGGCCAGCGC
1801  TGGTACGGAG CTGCATCCAT GAAATATCCG CAAACCCCTT TCAGTCGAGA ACACAACATG
1861  ATCGAGCGCA TACCCGAATT CTGCNTTCGC CGACGTAACT GGGTGGTCGG CGTGCTGCTT
1921  CTGCTCACGA TGGTGCTGAG CTGCTTCGCC CTGCATATCG ATGTCCGTAC CGTATTCGAG
1981  GACATGTTGC CCTCGCGCCA CGAGTACGTG AAAACCCACG AGAAGTTCAA GGACACCTTT
2041  GGCGGTTCGA ACATGGTCAC CATCATGTTC GAAGTGGATC AGGGCGAGAT CTTTCAGACT
2101  GCTGTGCTGG ATAAGGTTCG CACCGTCACC CTGGGCCTGG GTGAAGTGTC TGCGGTCAAC
2161  CAGTACCAGA TCACTTGCCT GGCCTCGAAG AAGCTCAAGG AGGTGCGCGC CTCTACCGCC
2221  GGCATCGAAA GCCGTTCGCT GATGTGGCCG GATCTACCGG CTAGTGCCGA GGATATGGCC
2281  GAGCTCAAGC AGGCAGTGTT GCGCAACCCG TTGGTCTACG GGCCGTACGT GTCGAAGGAT
2341  CTGCAGGCCA CGCTGGTTAC CGTGGATTTT ATCGACCAGC AGGTGGATTA CGCTACGGTC
2401  TTCCATCAGA TCCGCGAACT GATCGGCAAG GTCGATGACG GCAGCGTGAG CATCCGCGTG
2461  GTGGGTGATC CGATCCTCTA CGGCTGGGTG GGGCACTACC TGCCGGAGAC CATGCAACTG
2521  GTGGCCGCTG CGCTGCTGTT GACCCTGGTG ATGCTCTTCG CCCTGCTGCG TACCTGGCGC
2581  GGCATTACCC TACCGCTGCT GGCCGGCGTG GTCAGCGCCA TCTGGGCGCT GGGTATCTGC
2641  CGCCTGCTAA ACATCCATTT CGAGCCGCTG GTGATCGTGG TCGCCATGCT GATCACCTCG
2701  CGGGCCGTGT CGCATTCGGT ACAGATCGTC AATCGCTTTG ATGACGAGCT GCAGCTGTTG
2761  CCGCCTGGTT TCGATGTCTC GCGCATAGCT GCGCGGGTGG CTCTGTCGGA CCTGTTCCGC
2821  CCGGGCATGC TGGGGGTGAT TGCTGATGCC GCGTGCATGG CGGTTGTGGC GCTGAGCCCG
2881  ATCCCGATGC TACAGAAGCT CACGGTGCTA GCGGTAGTCT GGGTGCTGAC CCTGACGGTC
2941  AGTGCGGTGA TCCTCACCCC GGTGATGCTG TCCTACATCC GCAACCCCCA CAGCATGGCC
3001  CATCCGTTCG ACTGCCTGCC GGCGCTGCNC AAGGTGCTTC GACCTGGCGG TACGGGTCAA
3061  GCCTGTCGCG CTCGCGCTAT GTGGTTCTGG GTTTCAGCCT GTTGGCAANT GGTNTNCGCC
3121  GGGATCTA
```

**Figure Appendix IV-2. Sequence 1, DNA sequence data generated from pC1, pNHQ83 & pNHQ84**

# APPENDIX V. RESTRICTION SITES OF SEQUENCE 1

## Predicted restriction analysis on Sequence 1 (1-3128)

| Enzyme | Number | Site |
|---|---|---|
| AatII | 1 | 1610 |
| AccI | 4 | 90 1577 2315 3113 |
| AccII | 18 | 624 827 840 847 1334 1496 1733 1995 2207 2414 2456 2579 2700 2781 2793 2851 3068 3074 |
| AcyI | 6 | 18 158 384 1080 1607 1769 |
| AflIII | 2 | 1982 2327 |
| AluI | 12 | 1091 1197 1231 1565 1810 1940 2193 2283 2748 2754 2788 2898 |
| Alw26I | 2 | 2781 2500 |
| AlwNI | 3 | 507 516 1329 |
| ApaBI | 4 | 1330 2500 2758 3034 |
| ApaI | 2 | 45 267 |
| AsuI | 31 | 16 41 42 82 91 114 216 263 264 277 286 287 288 378 383 480 504 513 543 596 904 940 989 1015 1157 1532 2133 2320 2702 2808 2997 |
| AsuII | 2 | 2047 2069 |
| AvaI | 11 | 44 85 179 241 290 386 452 657 1252 1372 2820 |
| AvaII | 11 | 16 82 91 216 287 378 504 513 940 1157 2808 |
| BalI | 2 | 1366 1559 |
| BamHI | 1 | 1412 |
| BanI | 5 | 192 383 628 1059 1768 |
| BanII | 8 | 45 84 267 290 291 314 2285 2878 |
| BbeI | 1 | 1772 |
| BbvI | 22 | 403 509 854 938 1206 1335 1724 2453 2763 852 864 1089 1802 1906 1932 2519 2524 2557 2587 740 2780 3016 |
| BbvII | 3 | 467 1696 2397 |
| BclI | 2 | 1296 2690 |
| BglI | 3 | 262 855 2623 |
| BglII | 1 | 2087 |
| Bpu1102I | 2 | 1936 2872 |
| BsaHI | 6 | 18 158 384 1080 1607 1769 |

| | | |
|---|---|---|
| BsaOI | 11 | 90 94 133 831 934 1048 1141 1314 1383 1410 1782 |
| Bsc91I | 3 | 467 1696 2391 |
| BsiI | 2 | 1999 2017 |
| BsmI | 2 | 324 2712 |
| Bsp1286I | 17 | 19 20 45 85 86 94 95 127 256 267 290 291 381 943 1416 1459 1468 |
| Bsp1407I | 1 | 1162 |
| BspMI | 4 | 515 2507 501 2370 |
| BssHII | 2 | 845 2205 |
| Bst71I | 22 | 403 509 854 938 1206 1335 1724 2453 2763 849 861 1086 1799 1903 1929 2516 2521 2554 2584 2737 2777 3013 |
| BstEII | 6 | 89 123 252 1542 2055 2355 |
| BstNI | 28 | 97 121 182 200 332 415 434 502 517 605 878 1041 1170 1353 1387 1432 1469 1525 1582 1722 1738 2131 2137 2179 2545 2574 2765 3044 |
| BstXI | 1 | 2517 |
| Cfr10I | 15 | 58 195 284 320 478 891 929 1319 1461 1693 1765 2218 2256 2602 3018 |
| CfrI | 15 | 87 299 480 1311 1364 1380 1557 1589 1763 1779 1791 2245 2276 2522 2600 |
| ClaI | 2 | 799 1958 |
| Csp45I | 2 | 2047 2069 |
| CspI | 1 | 91 |
| DdeI | 6 | 3 28 472 789 1936 2872 |
| DpnI | 30 | 340 373 738 802 822 885 973 1038 1047 1298 1414 1420 1427 1595 1861 2078 2089 2170 2252 2339 2410 2422 2468 2473 2674 2692 2725 2881 2950 3124 |
| DraII | 10 | 16 41 82 91 263 286 287 504 513 1532 |
| DraIII | 1 | 3033 |
| DrdI | 1 | 511 |
| EagI | 3 | 1311 1380 1779 |
| Ecl136II | 1 | 2283 |
| Eco31I | 1 | 2500 |
| Eco47III | 1 | 1798 |
| Eco52I | 3 | 1311 1380 1779 |
| Eco56I | 5 | 320 1765 2218 2602 3018 |
| EcoHI | 26 | 16 31 47 48 88 89 244 245 288 293 294 334 389 390 427 482 660 661 910 971 1255 1256 2823 2824 2961 3122 |

| | | |
|---|---|---|
| EcoICRI | 1 | 2283 |
| EcoNI | 3 | 133 184 1333 |
| EcoRI | 1 | 1876 |
| EcoRII | 28 | 95 119 180 198 330 413 432 500 515 603 876 1039 1168 1351 1385 1430 1467 1523 1580 1720 1736 2129 2135 2177 2543 2572 2763 3042 |
| EcoRV | 1 | 1776 |
| EheI | 1 | 1770 |
| EspI | 2 | 1936 2872 |
| Fnu4HI | 52 | 28 154 168 191 194 197 286 347 384 392 400 407 498 563 566 590 838 843 861 873 924 927 1022 1051 1071 1098 1195 1209 1321 1324 1383 1438 1713 1782 1811 1915 1941 2442 2525 2528 2533 2566 2580 2596 2640 2665 2749 2752 2761 2789 2849 3025 |
| FnuDII | 18 | 624 827 840 847 1334 1496 1733 1995 2207 2414 2456 2579 2700 2781 2793 2851 3068 3074 |
| FokI | 11 | 1131 1185 1357 1599 1717 453 1800 2437 2638 2962 2987 |
| HaeII | 13 | 199 387 565 861 960 1329 1662 1772 1800 2618 2629 2872 3025 |
| HaeIII | 29 | 43 265 399 545 597 906 990 1017 1313 1351 1366 1382 1523 1534 1559 1591 1765 1781 1793 2135 2182 2247 2278 2322 2347 2524 2602 2704 2998 |
| HgaI | 5 | 472 764 1088 1640 1563 |
| HgiAI | 2 | 36 2285 |
| HhaI | 33 | 491 497 570 626 656 842 847 860 959 1289 1328 1334 1503 1623 1661 1712 1735 1771 1799 1868 1997 2207 2303 2532 2579 2617 2628 2783 2793 2871 3024 3070 3076 |
| HindII | 4 | 91 1578 2157 2540 |
| HinfI | 9 | 17 175 362 383 411 450 770 1245 1317 |
| HinP1I | 33 | 489 495 568 624 654 840 845 858 957 1287 1326 1332 1501 1621 1659 1710 1733 1769 1797 1866 1995 2205 2301 2530 2577 2615 2626 2781 2791 2869 3022 3068 3074 |
| HpaII | 28 | 86 242 291 321 387 658 892 907 930 954 968 1201 1253 1275 1314 1453 1462 1694 1766 2219 2248 2257 2503 2603 2821 2959 3019 3119 |
| HphI | 15 | 2152 2476 2559 2682 2847 2958 2973 320 437 938 1588 2049 2118 2685 2946 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| MaeI | 2 | 2261 | 2908 | | | | | | | |
| MaeII | 6 | 1009 | 1218 | 1607 | 1893 | 2006 | 2327 | | | |
| MaeIII | 13 | 2 | 89 | 123 | 197 | 252 | 382 | 436 | 944 | 1542 |
| | | 1894 | 2055 | 2124 | 2355 | | | | | |
| MboI | 30 | 338 | 371 | 736 | 800 | 820 | 883 | 971 | 1036 | 1045 |
| | | 1296 | 1412 | 1418 | 1425 | 1593 | 1859 | 2076 | 2087 | 2168 |
| | | 2250 | 2337 | 2408 | 2420 | 2466 | 2471 | 2672 | 2690 | 2723 |
| | | 2879 | 2948 | 3122 | | | | | | |
| MboII | 6 | 472 | 1459 | 1701 | 2199 | 2391 | 2547 | | | |
| MnlI | 17 | 961 | 1002 | 1222 | 1621 | 2001 | 2193 | 2220 | 2485 | 2706 |
| | | 2962 | 1329 | 1335 | 1368 | 1674 | 1971 | 2193 | 2262 | |
| MscI | 2 | 1366 | 1559 | | | | | | | |
| MspA1I | 12 | 47 | 58 | 96 | 196 | 1002 | 1021 | 1565 | 1784 | 2527 |
| | | 2595 | 2667 | 2754 | | | | | | |
| MspI | 28 | 86 | 242 | 291 | 321 | 387 | 658 | 892 | 907 | 930 |
| | | 954 | 968 | 1201 | 1253 | 1275 | 1314 | 1453 | 1462 | 1694 |
| | | 1766 | 2219 | 2248 | 2257 | 2503 | 2603 | 2821 | 2959 | 3019 |
| | | 3119 | | | | | | | | |
| MstI | 3 | 1502 | 1622 | 2302 | | | | | | |
| NaeI | 5 | 322 | 1767 | 2220 | 2604 | 3020 | | | | |
| NarI | 1 | 1769 | | | | | | | | |
| NheI | 1 | 2907 | | | | | | | | |
| NlaIII | 12 | 646 | 1131 | 1821 | 1860 | 1986 | 2055 | 2067 | 2514 | 2688 |
| | | 2829 | 2859 | 2997 | | | | | | |
| NlaIV | 27 | 17 | 18 | 43 | 83 | 84 | 92 | 93 | 125 | 194 |
| | | 254 | 265 | 287 | 288 | 289 | 379 | 385 | 598 | 630 |
| | | 941 | 1025 | 1061 | 1318 | 1414 | 1457 | 1466 | 1533 | 1770 |
| NruI | 1 | 827 | | | | | | | | |
| NspBII | 12 | 47 | 58 | 96 | 196 | 1002 | 1021 | 1565 | 1784 | 2527 |
| | | 2595 | 2667 | 2754 | | | | | | |
| NspI | 5 | 160 | 353 | 1332 | 1986 | 2829 | | | | |
| PflMI | 5 | 167 | 225 | 1133 | 2382 | 2994 | | | | |
| PleI | 3 | 370 | 444 | 764 | | | | | | |
| PpuMI | 6 | 16 | 82 | 91 | 287 | 504 | 513 | | | |
| PssI | 10 | 19 | 44 | 85 | 94 | 266 | 289 | 290 | 507 | 516 |
| | | 1535 | | | | | | | | |
| PstI | 4 | 512 | 1102 | 2345 | 2753 | | | | | |
| PvuI | 1 | 1048 | | | | | | | | |
| PvuII | 2 | 1565 | 2754 | | | | | | | |
| RleAI | 1 | 3004 | | | | | | | | |
| RsaI | 10 | 705 | 1164 | 1804 | 1968 | 2005 | 2164 | 2326 | 2571 | 2720 |
| | | 3051 | | | | | | | | |
| SacI | 1 | 2285 | | | | | | | | |
| SalI | 1 | 1576 | | | | | | | | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| SapI | 2 | 2560 | 1442 | | | | | | | |
| Sau3AI | 30 | 338 | 371 | 736 | 800 | 820 | 883 | 971 | 1036 | 1045 |
| | | 1296 | 1412 | 1418 | 1425 | 1593 | 1859 | 2076 | 2087 | 2168 |
| | | 2250 | 2337 | 2408 | 2420 | 2466 | 2471 | 2672 | 2690 | 2723 |
| | | 2879 | 2948 | 3122 | | | | | | |
| SciI | 1 | 1374 | | | | | | | | |
| ScrFI | 53 | 14 | 29 | 45 | 46 | 86 | 87 | 97 | 121 | 182 |
| | | 200 | 242 | 243 | 286 | 291 | 292 | 332 | 387 | 388 |
| | | 415 | 425 | 434 | 480 | 502 | 517 | 605 | 658 | 659 |
| | | 878 | 908 | 969 | 1041 | 1170 | 1253 | 1254 | 1353 | 1387 |
| | | 1432 | 1469 | 1525 | 1582 | 1722 | 1738 | 2131 | 2137 | 2179 |
| | | 2545 | 2574 | 2765 | 2821 | 2822 | 2959 | 3044 | 3120 | |
| SduI | 10 | 36 | 45 | 84 | 267 | 290 | 291 | 314 | 2285 | 2495 |
| | | 2878 | | | | | | | | |
| SfaNI | 12 | 475 | 1632 | 1822 | 2231 | 2459 | 1109 | 1163 | 1488 | 2540 |
| | | 2835 | 2876 | 2954 | | | | | | |
| SmaI | 5 | 243 | 292 | 659 | 1254 | 2822 | | | | |
| SphI | 1 | 2829 | | | | | | | | |
| SplI | 1 | 2324 | | | | | | | | |
| SpoI | 1 | 827 | | | | | | | | |
| SrfI | 1 | 2822 | | | | | | | | |
| SstI | 1 | 2285 | | | | | | | | |
| StyI | 6 | 180 | 259 | 280 | 302 | 536 | 3029 | | | |
| SunI | 1 | 2324 | | | | | | | | |
| TaqI | 20 | 753 | 799 | 1373 | 1577 | 1604 | 1845 | 1862 | 1958 | 1976 |
| | | 2047 | 2069 | 2185 | 2225 | 2332 | 2372 | 2432 | 2660 | 2771 |
| | | 3008 | 3039 | | | | | | | |
| ThaI | 18 | 624 | 827 | 840 | 847 | 1334 | 1496 | 1733 | 1995 | 2207 |
| | | 2414 | 2456 | 2579 | 2700 | 2781 | 2793 | 2851 | 3068 | 3074 |
| Tth111I | 1 | 215 | | | | | | | | |
| Tth111II | 2 | 297 | 2302 | | | | | | | |
| XcmI | 8 | 101 | 137 | 1070 | 1130 | 1176 | 1279 | 2517 | 2625 | |
| XhoI | 1 | 1372 | | | | | | | | |
| XhoII | 11 | 9 | 81 | 311 | 820 | 1036 | 1412 | 2087 | 2250 | 2337 |
| | | 2408 | 3122 | | | | | | | |
| XmaI | 5 | 241 | 290 | 657 | 1252 | 2820 | | | | |
| XmaIII | 3 | 1311 | 1380 | 1779 | | | | | | |
| XmnI | 1 | 143 | | | | | | | | |
| XorII | 1 | 1048 | | | | | | | | |

Non cutting enzymes:

| | | | | | |
|---|---|---|---|---|---|
| Acc65I | AccIII | AclI | AflII | AgeI | AhaIII |
| Alw44I | ApaLI | AscI | Asp718I | AvrII | BspHI |

| | | | | | |
|---|---|---|---|---|---|
| BspMII | BstD102I | Bsu36I | CvnI | DraI | Eam1105I |
| Eco57I | Eco72I | FseI | HindIII | HpaI | I-PpoI |
| KpnI | MfeI | Mlu113I | MluI | MseI | MstII |
| NcoI | NdeI | NotI | NsiI | PacI | PinAI |
| PmaCI | PmeI | SacII | SauI | ScaI | SfiI |
| SgrAI | SnaBI | SpeI | SspI | SstII | StuI |
| SwaI | VspI | XbaI | | | |

# APPENDIX VI. ORFS IN SEQUENCE 1

Potential open reading frames in Sequence 1(1-3128).

| Strand | RF | AA Num | Position | Sequence |
|---|---|---|---|---|
| Plus | 1 | 699 | 1030-3129 | agcggctccaATGgcaag... |
| Plus | 3 | 161 | 780-1265 | gactcgccctATGtcaaa...tggcgTAGccatc |
| Plus | 3 | 141 | 222-647 | gantggnncaATGggtgg...ggacaTGAatatc |
| Plus | 2 | 135 | 1013-1420 | ctggcgacgtATGggcca...gatccTGAtcggc |
| Plus | 1 | 120 | 1-363 | ...cgaaaTGAgtcgg |
| Plus | 2 | 107 | 1499-1822 | cagttccgcgATGcgcag...atccaTGAaatat |
| Minus | 3 | 252 | 1173-1931 | ggacatcgatATGcaggg...tccggTAGctgcc |
| Minus | 2 | 156 | 2660-3130 | gcactcagggATGcgtct... |
| Minus | 1 | 146 | 1063-1503 | cacttcgaacATGatggt...gtcccTGAtgcgc |
| Minus | 1 | 120 | 271-633 | cacaaccgccATGcacgc...gcaggTAGtgccc |
| Minus | 3 | 109 | 2802-3131 | ttccnggtgaATGccggc... |
| Minus | 2 | 57 | 1313-1486 | tatttcatggATGcagct...accgcTAAtgaac |